Relative Activities of Free and Esterified Vitamin D

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ABSTRACT

The saponification of fish liver oils may increase the activity of the vitamin D. In some cases the increases reached 100 per cent. That the increases were due to different activities of the esterified and free forms of the vitamin was demonstrated by a sample of vitamin D_2 palmitate undergoing a 250 per cent increase in activity upon saponification. Halibut liver oils showed anomalous behaviour in this respect, saponification resulting in either increased or unaltered activity, depending on the previous history of the material.

In a study of the effect of irradiation on various esters of ergosterol, Windaus and Rygh (1928) observed that, while both the unsaponified irradiation product of ergosteryl palmitate and the saponified material had antirachitic activity, the latter was the more active. Hickman (1937) showed that the greater part of the vitamin D in cod liver oil was in the form of esters, of which there appeared to be several, the remainder being present as the free vitamin. The nature of his work was such, however, that it could not reveal any possible differences between the activities of the free and esterified forms of the vitamin, since the antirachitic potencies of the various fractions isolated were used as the criteria for their vitamin D content. Chalmers and Biely (1940) examined two fish oils, prepared from pilchards and from salmon, and found that in each a part of the vitamin D was present in the ester form, and part as the free sterol. Since some vitamin concentrates from fish oils are prepared by saponification, while in other cases the concentration is accomplished by methods not involving saponification, it appeared that comparisons between the activities of the naturally-occurring vitamin D in various fish oils and those of the same materials after saponification would be of value.

A number of such comparisons have now been made in these laboratories and, in addition, some vitamin D_2 palmitate has been synthesized from calciferol, allowing a determination of the relative antirachitic activities of the pure calciferol, the synthetic ester and its hydrolysis product.

MATERIALS AND METHODS

With the exception of the dogfish liver oil and the halibut liver oils, all the samples used in the first part were commercially prepared oils. The dogfish liver oil was prepared in the laboratory by grinding the fresh livers and decanting the

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separated oil after a short period of settling. Some of the samples of halibut liver oil were prepared by alkali digestion of the livers, while others were prepared by solvent extraction. When ether was employed as the solvent it was reduced before use by distillation over alkaline ferrous sulphate, and tested for the presence of peroxides by the method of Dietze (1915). The herring and pilchard oils were prepared on an industrial scale by cooking the whole fish with live steam, pressing the cooked material in a continuous screw press and separating the oil from the rest of the press liquor by settling. The salmon oil was prepared from salmon cannery waste by the same method. The remaining oils were prepared by alkali digestion and centrifuging.

The oils were saponified by refluxing samples of from 1 to 5 g. with one-half the equivalent volume of 50 per cent potassium hydroxide and 10 volumes of aldehyde-free alcohol for one hour. The unsaponifiable residue containing the vitamin was recovered from the diluted soaps by two extractions with freshly-reduced ether. The combined extracts were washed with dilute alkali to remove the soaps they contained, and then with distilled water until neutral. The solvent was distilled off and the residue taken up in corn oil for administration to the animals.

The vitamin D_2 palmitate was prepared as follows. A 30-mg. sample of calciferol and 100 mg. of palmityl chloride were dissolved in 5 cc. of anhydrous pyridine and the solution was allowed to stand at room temperature for 2 weeks. It was then gently warmed for 15 minutes, diluted with water and extracted several times with ethyl ether. The combined extracts were washed twice with dilute sodium carbonate and then with distilled water until neutral. The ether solution was divided into two parts and the solvent distilled off from each separately. The residue from one part was taken up directly in corn oil; the other residue was saponified, and the vitamin extracted from the saponified material was then dissolved in corn oil.

The procedure followed in carrying out the vitamin D assays was that described in the First Supplement to the U.S. Pharmacopoeia XI (1937).

RESULTS

Miscellaneous Fish Oils

It is noteworthy that the increases in vitamin D activity upon saponification (table I) varied from zero to 100 per cent. From this it would appear that the relative amounts of the vitamin in the combined and free forms were markedly different in the various oils tested. Although the oils studied included samples from fish of widely different degrees of skeletal calcification, no relationship was found between the increase in potency of the oil and the group into which the fish fall with respect to their degree of calcification. The liver oil of the dogfish, a fish with a cartilaginous skeleton, showed an increase of 50 per cent, while the liver oils of the percomorphs, fishes with much higher skeletal calcification, showed increases of from zero to 100 per cent. Nor, apparently, are the increases confined only to liver oils; the vitamin D of both pilchards and herring showed enhanced activity after saponification, and although both these oils were prepared from the whole fish, most of the vitamin D in each is derived from the flesh.

The vitamin D in the samples of salmon and swordfish oil examined was apparently all in the free state, since no increase in antirachitic activity occurred in either when saponified. The results obtained by Chalmers and Biely (1940) differed from the above in that they found one-third of the vitamin D in a sample of salmon oil to be in the ester form.

TABLE I. Vitamin D potencies of various fish oils, before and after saponification, expressed in terms of 1 gram of the original oil.

Oil	Original oil (I.U./g.)	After saponification (I.U./g.)	Percentage increase
Herring	31	58	87
Pilchard	41	64	56
Salmon	115	115	0
Dogfish liver	10	15	50
Black cod liver	1,100	1,630	48
Ling cod liver	2,300	4,600	100
Albacore liver	20,000	40,000	100
Bluefin tuna liver	27,200	46,300	70
Striped tuna liver	29,600	41,800	41
Swordfish liver	8,000	8,000	0

HALIBUT LIVER OIL

In the experiments with halibut liver oil a peculiar phenomenon was observed. When a sample of the freshly produced oil was saponified, the unsaponifiable fraction exhibited in most cases a considerably higher antirachitic potency than that of an equivalent quantity of the original natural oil, vitamin D potencies of the unsaponifiable fractions being expressed, as before, in terms of one gram of the oil from which they were prepared. When, however, a sample of the same original oil was allowed to stand for 2 weeks or longer in a refrigerator at 3°C. and then saponified, the antirachitic potency of the unsaponifiable fraction showed no such increase, the value for both it and the stored oil being the same as that of the unstored oil. The experimental data obtained (table II) indicate that the lower activity of the unsaponifiable fraction from the stored oil is related to the freshness of the livers at the time the oil is extracted.

From table II it can be seen that, with the exception of lot 3, all the samples of fresh halibut livers yielded oils whose vitamin D had greater activity after saponification than before. The samples which showed enhanced activity after saponification and which were subsequently stored at 3°C. displayed the phenomenon already described, namely no increase in activity upon saponification of the oil after it had been thus stored for 2 weeks or longer. Oils prepared by either solvent extraction or alkali digestion of the cooked livers behaved similarly in respect to the above phenomena.

To determine whether a *stored unsaponifiable fraction* prepared from a *fresh oil* would exhibit the same activity as that oil, just as did a fraction prepared from a *stored oil*, a further experiment was conducted. The unsaponifiable matter from

TABLE II. Vitamin D potencies of halibut liver oils (variously extracted and stored), before and after saponification, expressed in terms of 1 gram of the original oil.

Lot no. of halibut livers	Nature of livers and method of preparing oil	Storage of oil before saponification		Vitamin D potency	
		Time (weeks)	Temp. (°C.)	Natural oil (I.U. per g.)	After saponification (I.U. per g.)
1	Fresh livers. Alkali digestion.....	0		2,900	4,000
	" " " "	2.5	+ 3	2,900	2,900
2	Fresh livers. Alkali digestion.....	0		700	1,000
	" " " "	4.5	+ 3	700	700
3	Fresh livers. Steaming and extraction with ethyl ether.....	0		1,500	1,500
4	Fresh livers. Steaming and extraction with petroleum spirits.....	0		4,000	8,000
	" " " " "	2	+ 3	4,000	4,000
5	Fresh livers. Steaming and extraction with petroleum spirits.....	0		3,100	3,700
	" " " " "	8	−20	3,100	3,700
	" " " " "	8	*	1,700	1,700
6	Fresh livers. Steaming and extraction with ethyl ether.....	0		880	1,400
	" " " " "	2	−20	880	1,400
7a	Fresh livers. Steaming and extraction with ethyl ether.....	0		1,200	1,700
	" " " " "	3	+ 3	1,200	1,200
7b	Same livers stored 2 weeks at +3°C., then steamed and extracted with ethyl ether.....	0		1,700	1,700
	" " " " "	3	+ 3	1,700	1,700

*Stored at room temperature in clear glass bottle on open shelf.

a sample of oil freshly prepared from lot 6 of livers was dissolved in corn oil in amount representing a 1:50 dilution of the original oil, and kept in a clear glass bottle on an open shelf at room temperature for 4 weeks. For comparison a sample of the same halibut liver oil was similarly diluted and stored. The potency of the freshly prepared oil was 880 I.U. per gram while the unsaponifiable fraction had an equivalent potency of 1400 I.U. (table II). After storage the corresponding potencies were 680 and 1190 I.U. (not shown in table). Thus even the drastic conditions of storage at room temperature and exposed to daylight in a clear glass container, which caused the oil to decrease in potency from 880 to 680 I.U., did not cause the potency of the saponified vitamin (1190 I.U.) to fall to the level of that of the natural vitamin in the freshly prepared oil (880 I.U.).

The question naturally arose as to what the relative potencies of the oil and its unsaponifiable fraction would be if the livers from which the latter was prepared had been first stored for several weeks at 3°C. In order to investigate this point one large lot of fresh livers (no. 7) was minced, thoroughly mixed, and divided into two parts. From one part (7a) the oil was extracted at once; the other (7b) was allowed to stand in the refrigerator for 2 weeks before extraction of the oil. The vitamin D in the oil prepared from the former displayed, as can be seen from table II, the phenomenon described; namely, while the fresh oil had a potency of 1200 I.U. per gram the potency of its unsaponifiable fraction showed, in equivalent amount, an enhancement to 1700 I.U. However, after storage of the oil for 3 weeks at 3°C. the potency of the unsaponifiable fraction then prepared from it showed no enhancement above the original 1200 I.U. value of the oil. On the other hand, the oil from the aged livers had, both before and after 3 weeks subsequent storage at 3°C., a potency of 1700 I.U. per gram and the potencies of the unsaponifiable fractions prepared therefrom in neither case exhibited any increase.

It is hoped that storage experiments now in progress will assist in explaining these phenomena.

Synthetic Vitamin D_2 Palmitate

Neither the vitamin D_2 palmitate which was synthesized nor its hydrolysis product was isolated in the pure form. They were fed to the animals in amounts based on the quantity of calciferol originally taken for the preparation of the ester. When thus tested the pure calciferol, the synthetic palmitate and the unsaponifiable fraction of the latter showed the following relative antirachitic potencies: calciferol, 10.0 ; vitamin D_2 palmitate, 1.0 ; hydrolysed vitamin D_2 palmitate, 2.5.

It is evident that there was considerable loss of vitamin D in the process of preparing the ester. However, since the hydrolysed vitamin D had two and a half times the antirachitic activity of the ester, it is clear that the decrease in activity during the preparation of the ester was not due entirely to destruction, but at least in part to a difference in activity between the ester and the free vitamin. As no attempt was made to separate from the synthetic palmitate any vitamin D which might have remained non-esterified, it is thus possible that the material designated as vitamin D_2 palmitate may actually have contained some free vitamin D; but this experiment shows that the ratio of the antirachitic activities of the free vitamin D_2 and the palmitate is at least 2.5 to 1.0.

The difference in activity found here substantiates that reported by Windaus and Rygh (1928). Furthermore, it shows that the differences in relative antirachitic potency found when various natural oils were saponified were due, at least in part, to differences in activity of the naturally-occurring vitamin D esters and their hydrolysis product.

DISCUSSION AND SUMMARY

While the vitamin D of some fish oils showed, upon saponification, no change in antirachitic activity, that of others showed a marked increase. Such increases,

which ranged as high as 100 per cent, showed no relationship to the group into which the fishes fell with respect to their degree of skeletal calcification. The increases do not appear to be confined to the vitamin D from any organ.

Halibut liver oil exhibited a behaviour unique among the oils studied. The unsaponifiable residue of a freshly prepared oil had, on the basis of an equivalent amount of oil, a higher potency than the oil itself, but when the same oil was stored at 3°C. for 2 weeks or longer before being saponified, the unsaponifiable residue then prepared from it had the same relative potency as the oil from which it was prepared, which remained unchanged during the storage. By storage of the saponified vitamin D from the fresh oil, and of the oil from which it was prepared, both diluted sufficiently with corn oil to minimize any possible effect of constituents of the halibut liver oil which would be eliminated by saponification, it was found that the antirachitic activity of the unsaponifiable fraction of the fresh oil did not fall to the level of the unsaponifiable fraction prepared from a similarly stored oil. This indicated that, whatever change occurred in the oil during storage, causing the unsaponifiable residue subsequently prepared from it to show no relative increase in potency over the oil, this change was not simple destruction of the vitamin. It is suggested that, during its storage in the oil as an ester, the vitamin underwent isomerization to a form which, unlike that present in the fresh oil, was no more active in the free state than as the naturally-occurring ester.

When a part of one sample of halibut livers was allowed to stand for 2 weeks at 3°C. before the oil was extracted, both the oil obtained from it and the unsaponifiable fraction of the oil had the same relative vitamin D potency as the unsaponifiable fraction of the fresh oil prepared from fresh livers of the same lot, this potency being higher than that of the fresh oil itself. Subsequent storage of the oil from the aged livers for 3 weeks at 3°C. caused no change in the potency of the oil, and the unsaponifiable fraction prepared from it after this storage also had the same relative antirachitic potency.

During the synthesis of a sample of synthetic vitamin D_2 palmitate, considerable loss of vitamin D activity occurred, but the activity of the subsequently hydrolyzed ester was two and a half times that of the ester itself, showing that the ratio of activities of free vitamin D_2 and vitamin D_2 palmitate is at least 2.5 to 1.0.

ACKNOWLEDGEMENT

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Fish Oils. IX. Certain Fish Oils as Sources of Nutritionally Essential Fatty Acids.

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ABSTRACT

Pilchard oil and two fractions of salmon egg oil, despite their greater unsaturation, proved less effective than methyl linoleate in curing rats suffering from essential fatty acid deficiency. Herring oil, less unsaturated, was even less effective.

It was shown by Evans and Burr (1927, 1928) that rats do not thrive when reared on a diet containing no fat. The chief symptoms observed under such a dietary regime are a scaly condition of the skin, particularly on the hind paws, and retarded growth. Other symptoms observed less regularly are scaliness of the front paws, loss of hair, and change of the skin on the tail so that the latter appears ringed. In severe cases the males show prolapsis of the penis. Evans and Lepkovsky (1932a) clearly demonstrated that saturated fatty acids are entirely ineffective in curing the condition; and the same authors (1932b) found that oleic acid is also ineffective. The only fatty acids conclusively shown to be effective are linoleic (Burr and Burr 1930), linolenic (Burr, Burr and Miller 1932) and arachidonic (Turpeinen 1938). The last author reported that methyl arachidonate was considerably more effective than methyl linoleate. While arachidonic acid does not ordinarily occur in fish oils the latter generally contain other highly unsaturated fatty acids with twenty or more carbon atoms. These, however, are not necessarily as effective as arachidonic acid. This was demonstrated by Hume *et al.* (1938). They fed to rats on a fat-free diet the methyl ester of docosohexaenoic acid, a fatty acid of twenty-two carbon atoms and six double bonds occurring in cod liver oil. While it did promote weight increase it showed little or no action in curing the skin lesions. The same authors (1940) found that neither the methyl esters of the total fatty acids of cod liver oil nor cod liver oil itself had appreciable activity in either promoting weight increase or protecting against skin lesions.

In the present work the activities of salmon egg (ova) oil, pilchard oil, and herring oil have been compared with that of methyl linoleate in curing the symptoms of essential fatty acid deficiency in rats.

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PREPARATION OF MATERIALS TESTED

The salmon egg oil was prepared in the laboratory from the mature ova of sockeye salmon (*Oncorhynchus nerka*) by cooking and pressing. The separated oil, which remained semi-liquid at −20°C., was filtered at that temperature, the two fractions thus obtained being designated as "salmon egg oil, liquid fraction" and "salmon egg oil, solid fraction". Both were liquid at room temperature.

The pilchard oil and herring oil were commercial products prepared from the whole fish and subsequently cold-cleared at 5°C. Methyl linoleate was prepared from the liquid fatty acids of corn oil by the method of Rollett (1909).

The iodine values (Wijs 30 minutes) of these materials were as follows:—

Salmon egg oil		Pilchard oil	184.0
liquid fraction	209.1	Herring oil	128.1
solid fraction	192.0	Methyl linoleate	171.4

BIOLOGICAL TECHNIQUE

The rats were weaned when 21 days old and were placed on the following diet when from 24 to 30 days old: glucose (cerelose), 79%; casein, 12%; brewer's yeast, 5%; salts (McCollum No. 185), 4%.

Both the casein and the yeast were thoroughly extracted with a low-boiling petroleum fraction (naphtha) before incorporation in the diet.

In addition to this diet the animals were given supplements of vitamins A, D, and E. During the earlier part of the investigation these were prepared by saponifying a mixture of cod liver oil and wheat germ oil, extracting the unsaponifiable fraction and dissolving it in liquid petrolatum for feeding. The solution thus prepared contained in a 50-mg. dosage, which was given weekly, 150 U.S.P. units of vitamin A and the unsaponifiable matter from 20 mg. of wheat germ oil. The vitamin D content was not determined, but it was considered adequate since the rats did not show any symptoms of rickets when autopsied at the end of the test.

Later the method of preparing the supplement of vitamins A, D, and E was changed. Ethyl laurate was substituted for liquid petrolatum as the solvent; the unsaponifiable matter of dogfish liver oil was used in place of that from cod liver oil to supply the vitamin A; pure calciferol was used for the antirachitic agent, and the unsaponifiable matter of wheat germ oil was replaced by synthetic α-tocopherol. This new preparation was made up to contain in the weekly dosage 50 U.S.P. units of vitamin A, 28 international units of vitamin D, and 1 mg. of α-tocopherol.

A considerable length of time was required to produce the symptoms of the deficiency in the animals, but after from 2½ to 4 months on the above regime their weights had become stationary or were declining, and all showed marked scaliness of the hind paws. The other symptoms previously mentioned occurred intermittently among the animals.

When the rats showed stationary or declining weight continuously for two weeks, together with marked scaliness of the hind paws, they were given the test

TABLE I. Comparative effectiveness of certain fish oils and methyl linoleate in curing deficiency of essential fatty acids in rats.

Test material	Daily dose (mg.)	Sex	Wt. gain during test period (g.)	Scaliness of hind paws	Other symptoms ; remarks
Salmon egg oil, liquid fraction ..	10	♀	..	++	Died after 75 days on test
		♀	6	+	
		♂	0	++	Prolapsed penis and extensive denudation.
	25	♂	63	++	Ringed tail.
		♀	42	++	Ringed tail, some denudation.
		♂	43	+	
	50	♂	53	−	Complete cure in 100 days.
		♀	38	+	Ringed tail.
		♂	72	+	Ringed tail.
Salmon egg oil, solid fraction ...	10	♀	55	+++	Ringed tail, some denudation.
		♂	62	+++	Ringed tail.
	25	♀	58	+++	Ringed tail.
		♂	65	++	Ringed tail, prolapsed penis.
		♂	75	+++	Ringed tail.
	50	♀	56	+	
		♂	63	++	Ringed tail.
		♀	60	+	
Pilchard oil.....	10	♀	11	++	Ringed tail, some denudation.
		♂	36	+++	
	25	♀	43	++	
		♂	44	+++	Ringed tail, prolapsed penis.
		♂	42	+++	Ringed tail, prolapsed penis.
	50	♂	58	++	Ringed tail.
		♀	70	++	Ringed tail.
		♂	71	++	Ringed tail.
Herring oil.....	10	♂	5	+++	Died after 25 days on test; prolapsed penis and severely ringed tail.
		♂	4	+++	Died after 33 days on test; prolapsed penis and severely ringed tail.
Methyl linoleate .	10	♂	56	−	Complete cure in 50 days.
		♂	83	+	
	25	♀	62	−	Complete cure in 40 days.
		♀	80	−	Complete cure in 33 days.
	50	♀	75	−	Complete cure in 48 days.
		♀	97	−	Complete cure in 30 days.
		♂	105	−	Complete cure in 30 days.

materials. These were administered from specially made and calibrated dropping-pipettes either daily or every second day, depending on the amounts the animals were receiving. Unless death intervened the animals were kept on test for 100 days.

RESULTS

In recording the data (table I), when the hind paws were only slightly scaly the condition was designated as + ; moderate scaliness was designated as ++ ; and severe scaliness as +++. Unless otherwise stated, such data refer to the condition of the animals at the end of the test.

A considerable number of negative controls were kept. All lost weight by the time they died, showing acute dermatitis and in many cases other symptoms.

DISCUSSION

None of the materials tested were as effective as methyl linoleate in either promoting weight increase or curing the skin symptoms. Although the effectiveness of these fish oils in alleviating the scaliness of the hind paws appeared to follow the same order as their iodine values, their relative activities in promoting growth were not in the same sequence. Thus, 10 mg. of the solid fraction of salmon egg oil (iodine value 192.0) produced in the animals a greater average weight increase than did a similar amount of the liquid fraction (iodine value 209.1). Furthermore, the two animals receiving 10 mg. of pilchard oil (iodine value 184.0) daily also showed greater growth than the three receiving a similar quantity of the salmon egg oil liquid fraction.

Herring oil (iodine value 128.1), in the amount fed, was markedly less active than the other oils tested.

The fact that the activity of fatty acids in general in alleviating or curing the syndrome is not simply a function of their unsaturation, is well exemplified in the present work by the superior activity of methyl linoleate.

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ERRATUM

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Lines 5 and 4 from bottom should read

" . . . the dielectric constant of the solvent decreases and the interfacial tension between water and the solvent increases with decreasing eluting"

The Adsorption of Vitamin A from Fish Liver Oils

By Lyle A. Swain

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ABSTRACT

In experiments with fish liver oils, vitamin A alcohol was more readily adsorbed than it fatty acid esters or than triglycerides and other fatty acid esters. The vitamin A esters were less readily adsorbed than the triglycerides.

The Tswett adsorption column, invented in 1906 and revived a quarter of a century later, has come into frequent use in the separation and purification of the components of a mixture. Its efficacy lies in the fact that the adsorptive forces existing between a dissolved substance and an adsorbent may be varied by changes in the adsorbent, changes in the solvent or changes in the molecule of the dissolved substance.

Changes in the effectiveness of the adsorbent are possible either by exchanging one substance for another or by different treatments ("activations") of any one substance. Seventy-one materials were examined by this method and ten showed definite adsorption of vitamin A alcohol. (In this paper careful distinction is made between vitamin A esters and vitamin A in its alcoholic (saponified) form because of the difference, to be described, in their adsorption properties.) Of two samples of barium carbonate of different origin, one adsorbed vitamin A alcohol and the other did not (Swain 1941). The adsorption of pigments from pilchard oil by a British Columbia bentonite clay was greatly improved by digestion with hydrochloric acid, which is one method of activation (Brocklesby and Moore 1933).

Changes in the solvent modify the adsorption process because solvents differ greatly in their "eluting power", that is, their ability to remove an adsorbate from an adsorbing surface. In general they may be arranged in what has been named an "eluotropic series" (Trappe 1940), in which each has a feebler eluting power than any preceding it in the series. The order of effectiveness of the eluting solvents is independent of the adsorbate or adsorbent used. Trappe points out that in this series the dielectric constant of the solvent and the interfacial tension between water and the solvent both decrease with decreasing eluting power. Light petroleum has a very weak eluting power; in other words, it is a solvent from which substances are readily adsorbed. On the other hand, it is very difficult to adsorb substances from a methanol solution. A series of solvents of

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graded eluting power is obtained by adding increasing proportions of a solvent of high eluting power, *e.g.*, methanol, to a solvent lower in the eluotropic series.

The chemical structure of the adsorbate has an important effect upon the ease of its adsorption. The more polar it is, the more readily it is adsorbed. Unsaturated compounds are more readily adsorbed than their saturated homologues, and conjugation of the double bonds (*i.e.*, the specific molecular arrangement $-CH=CH-CH=CH-$) increases this tendency. This is clearly shown in the much studied series of carotenoid compounds. These carotenoids also demonstrate that increasing numbers of hydroxyl groups in a molecule increase the ease of adsorption of the molecule, and that a hydroxyl group is more conducive to adsorption than is unsaturation. Again, zeaxanthin, an alcohol, is more firmly adsorbed than is its palmitic ester. Similarly, free fatty acids may be removed by adsorption from an oil consisting of esters of the fatty acids, a fact which has industrial application in oil refining. In the last two examples the polar group is masked by esterification.

METHOD

The apparatus used in the experiments to be described was made of two pieces of glass tubing of equal length, 12 mm. and 8 mm. inside diameter, sealed together to make a tube 25 cm. long, the narrower bottom end being constricted. The adsorbent was packed uniformly into the smaller tube with a flat-ended rod to a depth of 10 cm., a wad of cotton inserted beforehand preventing loss of the adsorbent through the constriction. The eluent escaping from the column was caught in a periodically changed receiver. The vitamin A content of the eluents was determined by the antimony trichloride reaction, the colour intensity being determined by a Rosenheim-Schuster tintometer except in the last experiment described, where an Evelyn photoelectric colorimeter was used (conversion factor 1 I.U. = 1.28 B.U.). The passage of vitamin A through the column was readily followed by its characteristic greenish-yellow fluorescence in ultraviolet light. The weight of eluate (material eluted) was determined after evaporation of the solvent.

EXPERIMENTAL AND DISCUSSION

An attempt was made to separate vitamin A ester from glyceryl esters (oil) by adsorption. Liver oil of the dogfish (*Squalus suckleyi*) was used which had been washed with methanol to remove any vitamin A alcohol that might be present. Five ml. of a dilute solution of this oil in benzene, containing 11 mg. oil and 42 I.U. of vitamin A per ml., was passed through a column of alumina activated by heating at 400°C. for 40 hours (Krieger 1941). This was followed by passage of benzene to develop the column. From the data under (A) in table I it is seen that vitamin A appeared in the eluents immediately and was therefore not adsorbed. On the other hand the oil was delayed until the third eluent, and ceased at the seventh when only 10 per cent had escaped from the column. This means that the oil was adsorbed preferentially and some separation of it

had occurred, possibly on the basis of length of the fatty acid chains present or of their unsaturation. Only the least adsorbed fraction passed through in this experiment.

The same experiment was carried out using chloroform as solvent, the solution containing 10 mg. oil and 38 I.U. vitamin A per ml. The data given under (B) in table I again show an immediate passage of vitamin A esters. The low vitamin content of the first eluent and the low total amount of vitamin recovered probably were due to its partial destruction, an effect known to be readily caused in chloroform solution in the presence of light. Oil (residue) appeared in the eluent earlier when chloroform was used as solvent than when benzene was used, which agrees with the position of chloroform in the eluotropic series above benzene.

TABLE I. Adsorption of vitamin A esters and dogfish liver oil on alumina.

	Eluent (ml.)	Residue (mg.)	Oil (%, cumulative)	Vitamin A content (I.U.)	Vitamin A (%, cumulative)
(A) From benzene solution	2	0	0	51	24
	2	0	0	77	61
	2	0.2	0.4	45	82
	2	1.7	3.5	5	85
	2	2.9	8.7	1	85
	2	0.7	10.0	8	89
	2	0.1	10.0	0	89
(B) From chloroform solution	2	0.2	0.4	24	13
	2	0.5	1.4	74	52
	2	1.1	3.6	56	81
	2	1.6	6.8	trace	81

The presence of vitamin A in the initial eluents is to be particularly noted. It consisted of vitamin A esters only, because of the methanol washing. That the behaviour of vitamin A alcohol in an adsorption column is very different from that of vitamin A esters has been mentioned in the literature (Mead 1939; Hickman and Mees 1941). It is demonstrated in the following experiments.

A portion of liver oil freshly prepared from fresh halibut (*Hippoglossus stenolepis*) was saponified. The oil containing vitamin A esters and the unsaponifiable matter containing vitamin A alcohol, were separately dissolved in light petroleum (b.p. 35°-60°C.) to make solutions of equal vitamin potency (94 I.U. per ml.). Ten ml. of each solution was passed through identical columns of silicic acid. The columns were then developed, first with light petroleum and then with chloroform. Progress in the passage of the vitamin in each column is shown in table II. It can be seen that the vitamin A esters in the oil passed through the column with only slight adsorption. The first 4 ml. of the first eluent contained no vitamin A as indicated by lack of fluorescence. This delay was greater than with benzene or chloroform (A and B in table I) which are

TABLE II. Adsorption of vitamin A in the ester and alcohol form on silicic acid from light petroleum

	Solution of oil		Solution of unsaponifiable matter	
	Eluent (ml.)	Vitamin A content (I.U.)	Eluent (ml.)	Vitamin A content (I.U.)
Light petroleum	10	128	10	0
	2	300	10	0
	2	286	10	0
	2	179	..	...
	2	33	..	...
	2	5	..	...
	2	3	..	...
	2	0	..	...
Chloroform	10	0	10	0
	10	0	10	400
	10	0	10	368
	10	0	10	95
	10	0	10	8
	10	0	10	4
	10	0	10	3
	..	...	10	0
		Total 926		Total 881

stronger eluting solvents. On the other hand, vitamin A alcohol in the unsaponifiable matter was adsorbed strongly from light petroleum solution and was eluted completely by chloroform, a solvent higher in the eluotropic series. The vitamin A fluorescence showed that, after 30 ml. of light petroleum had passed through the column, the vitamin A alcohol had penetrated into the column only 3 mm. Chloroform carried the fluorescence down through the column immediately.

Since it might be argued that the difference in behaviour of vitamin A in the two solutions described at the beginning of the preceding paragraph was due to the presence of the oil in one, equal volumes of these solutions were mixed and passed through a column of silicic acid. The column was then washed with light petroleum, which left a brightly fluorescing zone at the upper surface. The top 5 mm. of adsorbent was scraped off and washed with methanol. A portion of the light petroleum eluent and of the methanol extract were each examined for their vitamin A alcohol content by partition of the vitamin between hexane and 82 per cent ethanol (Clausen at meeting of American Chemical Society at Cincinnati, in 1940). The vitamin A in the light petroleum was in the ester form and that in the methanol extract was in the alcohol form. The two forms of vitamin A were thus separated by the adsorption column in the presence of oil, which was therefore not a factor affecting the adsorption of vitamin A.

The experiments outlined above show that vitamin A esters cannot readily be separated from triglycerides by adsorption, but that vitamin A alcohol can easily be separated therefrom. A process was devised to hydrolyze vitamin A esters without converting the glyceryl esters to soaps. From the resulting product it is possible to separate vitamin A, now in the alcohol form, from the fatty acid esters which are also produced, just as in the experiment last described. A 2.0 per cent solution in benzene of dogfish liver oil treated by this method was passed through a column of alumina which had been activated by digestion with hydrochloric acid. Addition of the solution to the column was continued until the fluorescence of vitamin A reached the bottom of the column, to yield eluent 1 (table III). The receiver was changed and the column was washed with benzene containing 0.5 per cent ethanol. Possessing greater eluting properties than pure benzene, this solvent rapidly eluted vitamin A from the column almost entirely, and was caught in two almost equal portions (eluents II and III). A small volume of 95 per cent ethanol removed the last traces of fluorescence from the column, forming eluent IV. The vitamin A content, non-volatile residue

TABLE III. Composition of eluents

Eluent	Volume (ml.)	Vitamin content (I.U.)	Weight of residue (g.)	Vitamin potency (I.U. per g.)	Unsaponifiable (%)
I Benzene	97	470	1.629	290	0.06
II 0.5% ethanol in benzene	51	7230	0.181	39,900	
III " " " "	50	120	0.052	2270	91.0
IV 95% ethanol	10	160	0.129	1280	

and unsaponifiable matter of the eluents were determined and are given in table III. The fatty acid esters passed through the column without being adsorbed and appeared almost entirely in eluent I. The unsaponifiable matter was mostly adsorbed, since the residue of eluent I contained only 0.06 per cent unsaponifiable matter. The presence of ethanol displaced vitamin A alcohol almost completely from the column into eluent II which also contained about half of the unsaponifiable matter.

This experiment indicates the ease with which vitamin A alcohol can be separated from fatty acid esters, and the relative difficulty in separating it from the other components of the unsaponifiable matter of dogfish liver oil.

CONCLUSIONS

The difference in ease of adsorption of vitamin A alcohol and its esters has been mentioned in the literature, but experimental data have not come to the attention of the writer. The data above give justification for the statement that the alcohol form of vitamin A is much more easily adsorbed than its esters. It is also more easily adsorbed than are glyceryl and other esters of fatty acids. It

is apparently a little more readily eluted than some other components of the unsaponifiable matter of dogfish liver oil. Vitamin A esters are less readily adsorbed than are other components of dogfish liver oil.

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Rise and Fall of Bacterial Populations in Fish Muscle

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ABSTRACT

A direct bacterial count method applicable to fish muscle containing over approximately 1×10^5 bacteria per gram, with a modification employing preliminary incubation for muscle containing fewer bacteria, is described. Direct counts generally yielded similar and somewhat higher results than viable counts. Mixed bacterial populations of fish muscle generally exhibited the growth phases characteristic of a pure bacterial culture. There was absolutely no relation between direct (or viable) counts obtained subsequent to such incubation and the initial viable counts of the samples studied. Bacteria increased much more rapidly in excised fish muscle than in whole fish, and somewhat more rapidly in halibut than in salmon flesh.

Enumeration of viable bacteria in fish muscle has frequently been used as a means of ascertaining to what extent bacterial spoilage has proceeded therein. However, even when employed as a research method, the technique of making viable bacterial counts is time-consuming and requires a rather large number of manipulations. The method of determining the number of bacteria in fish muscle by direct microscopic observation (Tarr 1941) does not overcome the difficulties and errors due to sampling, preparation and grinding muscle samples, but it does eliminate the procedures of making dilutions, plating and incubating, which are inherent in any of the modifications of the technique of making viable bacterial counts. As far as can be ascertained there are no other published accounts of methods by which bacteria have been counted directly in fish tissues, though Haines (1937) states that similar techniques have been used in the case of meats, but does not give any direct references to these.

EXPERIMENTAL

In the experiments to be described, fish from a variety of sources were examined, their history after capture in most instances not being thoroughly known. However, it is practically certain that none of the fresh fish examined had been kept for more than 5 days in crushed ice subsequent to capture, and in some cases fish still in *rigor mortis* were used. The relatively low initial bacterial counts obtained with most samples are themselves indicative of freshness.

Whole, dressed fish were stored in crushed ice in a room at approximately 1.5°C., a single fish being removed for each determination and sampled by the

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technique previously described (Tarr and Bailey 1939). Prior to filleting, fish were washed thoroughly and the slime was removed. They were cut up under very clean conditions in order to reduce initial bacterial contamination.

In some experiments a number of small fillets weighing about 150 to 200 g. were stored separately in covered, sterile 600-ml. beakers, a single fillet being used for each analysis. This method gave rather irregular results, and so for most experiments about 1 kg. of fish flesh passed through a sterile domestic mincing machine was stored in a 2-l. covered beaker, the comminuted flesh being mixed thoroughly before sampling. At first, samples were stored at 1.5 ± 1.0°C.; but later, when special cabinets were made available, a temperature of 0.5 ± 0.5°C. was used.

Individual fillets were minced in a sterile petri dish with scissors prior to examination. Minced flesh was sampled directly, about 50 to 60 g. being transferred to a sterile petri dish. In cases where the flesh was incubated prior to making bacterial counts, about 50 g. was placed in a sterile petri dish and stored for 24 hours at 15 ± 0.5°C.

Bacterial counts were made on a 1 in 4 aqueous extract of minced muscle prepared as usual. Viable bacterial counts were made in duplicate by the "roll tube" method using double-strength "Bacto" nutrient agar containing 0.02 M. phosphate buffer and adjusted to pH 7.0 as substrate (Tarr and Bailey 1939). Direct, or total, bacterial counts were made by a technique practically identical with that used for milk (Breed and Drew 1916). The large amounts of tissue debris rendered attempts to count bacteria in unstained preparations useless.

After allowing the sand and heavy tissue debris to settle and removing samples for viable bacterial counts, duplicate 0.01-ml. portions of the aqueous extract were transferred to an ordinary microscope slide, each droplet being then spread over an area of approximately 1 sq. cm. by means of a sterile needle. The resulting films were dried on a level surface at about 40°C. and were then fixed by immersing them for 1 minute in 95 per cent ethyl alcohol. They were then stained with Loeffler's methylene blue for approximately 30 seconds, rinsed gently with water and dried as above. Treatment of the dry films with toluene in order to remove fat globules as in the case of milk was not found necessary.

The bacteria in these films appeared deep blue against a pale blue background and were counted by means of a standardized microscope equipped with an oil immersion objective and a 6x eyepiece containing an ocular micrometer. With the equipment employed it was found that a single microscope "field" corresponded to $1/(2.65 \times 10^6)$ g. of fish muscle. This meant that fish muscle containing 1×10^5 bacteria per gram would have only one organism in 26.5 microscope fields, or approximately four per 100 fields. It was obvious, therefore, that the method was liable to be rather inaccurate in this region unless an extremely large number of fields was counted, and could not be applied directly to fish with fewer than about 1×10^5 bacteria per gram. For such fish some modification of the method was necessary.

Attempts to concentrate the organisms in aqueous fish muscle extracts by centrifuging and then to count them did not prove satisfactory. The obvious alternative for fish muscle of low bacterial content was to incubate it for fixed time and temperature in order to facilitate multiplication of the organisms to a level at which direct counts could readily be made. After some preliminary trials it was found that good results could be obtained by incubating the minced muscle for 24 hours at 15°C., though, for reasons which will be given later, there proved to be no relation between bacterial counts obtained prior to and subsequent to such incubation.

It was found that there was often a tendency for slightly more bacteria to be concentrated along the margins of stained films, and this effect had to be taken into consideration when direct counts were made. Due to the very great variability in the number of bacteria to be counted in different samples, it was not found possible to count the same number of microscope fields for each sample. Thus in samples containing about 1×10^5 organisms per gram 100 fields in each of two duplicate films were counted, while in a sample containing about 200×10^6 organisms per gram only 10 fields in each of two films were counted. In the case of putrid fish muscle which had, for example, about $10{,}000\times10^6$ organisms per gram it was found necessary to dilute the extract ten-fold before satisfactory preparations could be obtained.

The accuracy of the method has not yet been determined. There are a number of possible sources of error in addition to those mentioned above, but it is rather doubtful if these are any more serious than those encountered in viable-count techniques.

Experiment 1

Six dressed chum salmon (*Oncorhynchus keta*) weighing from 3 to 3.5 kg. were obtained packed in crushed ice. Five were immediately re-iced and stored at 1.5°C. The remaining fish was filleted and a single small fillet placed

Table I. Increase in bacterial populations in stored chum salmon.

Storage period (days)	Whole fish stored in crushed ice		Individual fillets stored at 1.5°C.	
	Direct count (bacteria per g.)	Viable count (colonies per g.)	Direct count (bacteria per g.)	Viable count (colonies per g.)
0	.....	.....	0	2,700
1	0	16	0	740
2	.....	.....	0	1,800
3	.....	.....	0	8,600
4	.....	.....	200,000	43,000
5	0	720	0	9,200
7	.....	.....	51×10^6	3×10^6
10	0	5,200	35×10^6	37×10^6
13	93,000	320,000	880×10^6	$1{,}400\times10^6$
19	650,000	1.4×10^6	$18{,}000\times10^6$	$13{,}000\times10^6$

in each of ten beakers and stored at 1.5°C. Bacteriological analyses of both whole fish and fillets were carried out from time to time by the methods already described. The results (table I) showed that, during the period of examination, both viable and total bacterial counts were very much higher in the fillets than in the whole dressed fish. Similar results were obtained with haddock by Griffiths and Stansby (1934). It will be noticed that both in whole fish and fillets the increase in bacterial numbers was not consistent, indicating that the initial contamination had been variable as regards number and possibly type of organism. Where bacteria were present in sufficient numbers to be counted directly there was usually a reasonably close agreement between viable and direct counts, though the discrepancy was rather marked on the seventh day of sampling in the case of fillets.

EXPERIMENT 2

The rate of increase in bacterial numbers at 1.5°C. was followed in both individual fillets and minced muscle obtained from a 5-kg. defrosted halibut (*Hippoglossus stenolepis*). The results (table II) verified those of the previous experiment in showing that the increase was not very consistent in the case of isolated fillets. Much more consistent results were obtained with minced

TABLE II. Increase in bacterial populations in defrosted halibut muscle stored at 1.5°C.

Storage period (days)	Individual fillets		Minced muscle	
	Direct count (bacteria per g.)	Viable count (colonies per g.)	Direct count (bacteria per g.)	Viable count (colonies per g.)
0	0	1,000	0	1,200
1	0	270	0	860
2	0	490	0	1,700
3	0	510	0	1,500
4	0	4,200	0	1,300
6	1.4×10^6	740,000	530,000	23,000
8	0	2,400	400,000	290,000
10	61×10^6	54×10^6	16×10^6	47×10^6
11	.....	.....	28×10^6	51×10^6
13	14×10^6	14×10^6	330×10^6	240×10^6
15	.....	.....	$2{,}800\times10^6$	$1{,}500\times10^6$
16	.....	.....	$3{,}600\times10^6$	$2{,}600\times10^6$
20	$5{,}300\times10^6$	$2{,}900\times10^6$	$31{,}000\times10^6$	$21{,}000\times10^6$
27	.....	.....	$29{,}000\times10^6$	$20{,}000\times10^6$
32	.....	.....	$21{,}000\times10^6$	$16{,}000\times10^6$
83	940×10^6	$1{,}400\times10^6$	$1{,}900\times10^6$	$1{,}900\times10^6$

muscle, and in subsequent experiments only this form of muscle was employed. On the whole the rate of increase of bacteria was not as rapid in the individual fillets as in the comminuted flesh. In general, direct counts followed a trend similar to that of the viable counts, though normally, but not invariably, the

former were somewhat higher than the latter (figure 1). In the minced sample the phases characteristic of the growth of a pure bacterial culture (Buchanan 1918) were fairly well distinguished. Thus there was a well-defined initial stationary phase, a brief lag phase and then a prolonged phase of logarithmic increase. The phase of negative growth acceleration, the stationary phase and that of accelerated death were also evident. From the practical standpoint only the first three phases are likely to prove of interest since fish flesh becomes stale during the logarithmic phase.

EXPERIMENT 3

Minced muscle obtained from a 5-kg. fresh halibut was stored at 1.5°C., bacterial counts being made periodically. The results (table III) showed that in all instances the direct counts were slightly higher than the viable

TABLE III. Increase in bacterial populations in minced halibut muscle stored at 1.5°C.

Storage period (days)	Direct count (bacteria per g.)	Viable count (colonies per g.)
0	0	2,700
1	0	2,200
2	0	5,400
3	0	5,600
4	40,000	22,000
5	79,000	72,000
7	1.2×10^6	680,000
8	8.4×10^6	5.3×10^6
10	35×10^6	25×10^6
14	$6{,}900\times10^6$	$4{,}000\times10^6$
19	$27{,}000\times10^6$	$8{,}400\times10^6$
29	$19{,}000\times10^6$	$7{,}200\times10^6$
80	$6{,}400\times10^6$	$2{,}500\times10^6$

counts. However, both gave a closely similar measure of the rate of bacterial increase (figure 1), though the initial stationary growth phase was not as marked as in the case of the defrosted halibut used in the previous experiment.

EXPERIMENT 4

A comparison of both viable and direct bacterial counts on different samples of minced muscle from halibut, spring salmon (*Oncorhynchus tschawytscha*) and coho salmon (*O. kisutch*) stored at 0.5°C. was made both before and after incubating samples of the flesh for 24 hours at 15°C. The results are given in table IV and figure 2.

It was found that bacterial development was a good deal slower in the species of salmon than in the halibut. This may have been due to the oily nature of salmon flesh. In this connection it is of interest that Leudsen and

TABLE IV. Increase in bacterial populations (colonies per g. by viable count, bacteria per g. by incubating samples 24 hours at 15°C. (storage temperature 0.5°C.). B = Prior to incubation.

Kind of fish		Halibut							
Sample no.		1		2		3		4	
Storage period (days)	Treatment	Viable count	Direct count	Viable count	Direct count	Viable count	Direct count	Viable count	Direct count
0	B	2,700	0	4,700	0	3,500	0	7,600	0
	A	500,000	2.4×10^6	190,000	190,000	1.2×10^6	2.4×10^6	4.2×10^6	5.2×10^6
	A/B	190		40		340		550	
1	B	2,200	0	4,100	0	17,000	0	6,900	0
	A	4×10^6	6.9×10^6	2×10^6	2.2×10^6	42×10^6	85×10^6	17×10^6	33×10^6
	A/B	1,800		490		2,500		2,500	
2	B	5,400	0	3,200	0	24,000	80,000	12,000	0
	A	27×10^6	27×10^6	20×10^6	31×10^6	66×10^6	410×10^6	29×10^6	100×10^6
	A/B	5,000		6,200		2,800	5,100	2,400	
3	B	5,600	0	8,200	0	160,000	410,000	23,000	0
	A	50×10^6	94×10^6	14×10^6	28×10^6	640×10^6	$1,200\times10^6$	190×10^6	280×10^6
	A/B	8,900		1,700		4,000	2,900	8,300	
4	B	22,000	40,000	19,000	0	1.9×10^6	8.8×10^6	110,000	120,000
	A	610×10^6	930×10^6	96×10^6	92×10^6	..	..	$1,000\times10^6$	$1,700\times10^6$
	A/B	28,000	23,000	5,100		..	..	9,100	14,000
5	B	72,000	79,400	..	..	..	..	..	..
	A	..	..	..	..	..	..	..	..
	A/B	..	..	..	..	..	..	..	..
6	B	..	..	120,000	280,000	..	..	..	..
	A	..	..	$1,200\times10^6$	$1,200\times10^6$	..	..	..	..
	A/B	..	..	10,000	4,300	..	..	..	..
7	B	680,000	1.2×10^6	720,000	930,000	..	..	..	..
	A	..	..	..	..	..	..	..	..
	A/B	..	..	..	..	..	..	..	..
8	B	5.3×10^6	8.4×10^6	..	..	..	..	..	..
	A	..	..	..	..	..	..	..	..
	A/B	..	..	..	..	..	..	..	..
9	B	25×10^6	35×10^6	..	..	..	..	..	..
	A	..	..	..	..	..	..	..	..
	A/B	..	..	..	..	..	..	..	..
10	B	..	..	..	..	..	..	..	..
	A	..	..	..	..	..	..	..	..
	A/B	..	..	..	..	..	..	..	..
11	B	..	..	..	..	..	..	..	..
	A	..	..	..	..	..	..	..	..
	A/B	..	..	..	..	..	..	..	..

direct count) in stored halibut, spring salmon and coho salmon before and after A = After incubating 24 hours at 15°C. A/B = Ratio of A to B.

		Spring Salmon				Coho Salmon	
5		6		7		8	
Viable count	Direct count	Viable count	Direct count	Viable count	Direct count	Viable count	Direct count
1,300	0	2,700	0	3,300	0	6,000	0
150,000	190,000	310,000	280,000	36,000	80,000	2.4×10^6	7.7×10^6
120		110		11		400	
1,800	0	2,700	0	3,000	0	7,300	0
2.8×10^6	3.5×10^6	700,000	440,000	240,000	159,000	2.9×10^6	11×10^6
1,600		260		80		400	
1,700	0	2,100	0	2,900	0	4,900	0
7.3×10^6	11×10^6	2.7×10^6	2.2×10^6	140,000	120,000	8.3×10^6	57×10^6
4,300		1,300		48		1,700	
10,000	0	4,800	0	3,200	0	24,000	0
13×10^6	17×10^6	12×10^6	8.6×10^6	480,000	410,000	57×10^6	190×10^6
1,300		2,500		150		2,400	
15,000	53,000	5,400	0	3,100	0	11,000	0
200×10^6	340×10^6	22×10^6	24×10^6	1.2×10^6	2.5×10^6	37×10^6	260×10^6
13,000	6,500	4,100		390		3,400	
140,000	160,000	15,000	0	3,100	0	23,000	40,000
220×10^6	570×10^6	60×10^6	62×10^6	2.3×10^6	7.3×10^6	150×10^6	340×10^6
1,500	3,600	4,000		740		6,500	8,500
360,000	1.2×10^6	36,000	0	4,400	0	160,000	120,000
470×10^6	1300×10^6	74×10^6	67×10^6	6.3×10^6	13×10^6	390×10^6	650×10^6
1,300	1,100	2,100		1,400		2,400	5,400
..	..	..	39,000	9,600	0	350,000	420,000
..	..	180×10^6	200×10^6	37×10^6	39×10^6	$1,000\times10^6$	$2,000\times10^6$
..	..	..	5,100	3,900		2,900	4,800
..	..	..	..	17,000	0	1.9×10^6	7.3×10^6
..	..	..	..	50×10^6	67×10^6	$2,700\times10^6$	$5,500\times10^6$
..	..	..	..	2,900		1,400	750
..	..	410,000	400,000	33,000	0	..	..
..	..	280×10^6	470×10^6	80×10^6	106×10^6	..	..
..	..	680	1,200	2,400		..	..
..	..	3.4×10^6	2.9×10^6	180,000	290,000	..	..
..	..	..	..	320×10^6	360×10^6	..	..
..	..	..	..	1,800	1,200	..	..
..	..	..	..	1.5×10^6	880,000	..	..
..	..	..	..	$2,300\times10^6$	$2,700\times10^6$	..	..
..	..	..	..	1,500	3,100	..	..

Derlich (1940) showed that certain fish liver oils possess marked bactericidal properties. On the whole, however, the general shape of the bacterial growth curves was similar in both types of fish. Thus, prior to incubation the initial stationary, lag and logarithmic growth phases were usually evident. Subsequent to incubation the points obtained usually fell approximately along a straight line indicating that regular logarithmic increase had occurred during the incubation period. The points were in some cases rather scattered, but this was hardly unexpected due to the many possible sources of error in making the counts. As in preceding experiments, direct counts were normally somewhat higher than viable counts, though there were certain exceptions.

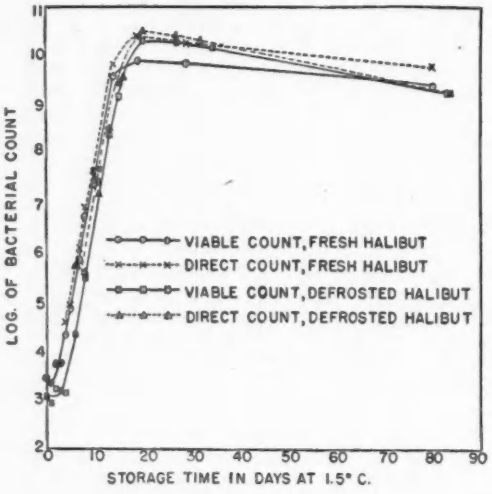
LOG. OF BACTERIAL COUNT
STORAGE TIME IN DAYS AT 1.5° C.
VIABLE COUNT, FRESH HALIBUT
DIRECT COUNT, FRESH HALIBUT
VIABLE COUNT, DEFROSTED HALIBUT
DIRECT COUNT, DEFROSTED HALIBUT

FIGURE 1. Rise and fall of bacterial populations in fresh and defrosted halibut muscle (Experiments 2 and 3).

From table IV it will be seen that the ratios (A/B) of the pre-incubation viable counts to those obtained subsequent to incubation for 24 hours at 15°C. were extremely variable. Thus in the case of different halibut samples examined these ratios varied from 40 to 550 at the commencement of the tests, and from 490 to 2500 after storage for 1 day at 0.5°C. In one instance a ratio of 28,000 was obtained after a sample had been stored for 4 days. This is probably largely due to the fact that during the first 1 to 3 days of storage the bacteria in freshly excised fish muscle either do not multiply or do so rather slowly (initial stationary and lag phases). On the other hand the *tendency to multiply* under more favourable temperature conditions greatly increases during this period, and hence the great variations in the ratios. Differences in growth rates of the various bacteria occurring in the samples would also have played some part in the effect observed. It is obvious from this result that it would

be impossible to predict the initial bacterial count of a given sample of fish from the direct (or viable) bacterial count obtained subsequent to incubation.

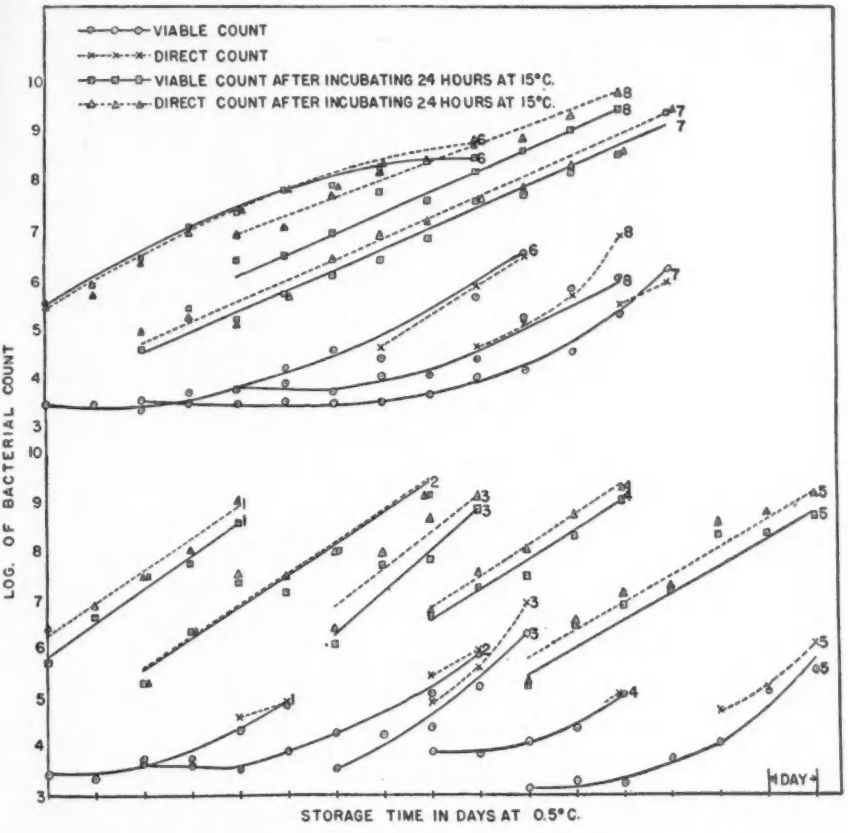
VIABLE COUNT
DIRECT COUNT
VIABLE COUNT AFTER INCUBATING 24 HOURS AT 15°C.
DIRECT COUNT AFTER INCUBATING 24 HOURS AT 15°C.
LOG. OF BACTERIAL COUNT
STORAGE TIME IN DAYS AT 0.5°C.
4 DAY

FIGURE 2. Increase in bacterial populations in fresh halibut (1 to 5), spring salmon (6, 7), and coho salmon (8) muscle, prior and subsequent to incubating for 24 hours at 15°C (Experiment 4).

DISCUSSION

On the whole direct counts were somewhat higher than viable counts, and in this respect the results were similar to those which Wilson (1922) obtained with pure bacterial cultures. Occasionally direct counts gave lower results than did viable counts. The reason for this was not clear, but it may have been due to the presence of small organisms not readily differentiated in stained preparations or simply to unavoidable sampling errors. There was reasonably good agreement between the methods even with samples containing some 1×10^5 bacteria per gram, and it would seem that, at least for fish muscle containing greater numbers of organisms, direct counts might prove as valuable

as viable counts. For fish muscle of bacterial content lower than 1×10^5 organisms per gram incubation would be necessary in order to raise the number of bacteria to a level at which direct counts could be made. Such incubation would yield a more reliable index of the keeping quality of the fish than would a more time-consuming and tedious initial viable count, for reasons already given.

One possible undesirable feature of the direct count lies in the fact that both viable and non-viable organisms are counted. Thus the direct count obtained in the case of a piece of fish muscle grossly contaminated with dead bacteria would naturally be much higher than the viable count. However, contamination which was heavy enough to influence the direct count would itself be indicative of unclean conditions.

The possible value of the direct count method in evaluating the bacterial quality of fish should be considered. The quality of fish containing over about 1×10^5 bacteria per gram could probably be determined fairly rapidly by it. On the other hand, for fish of lower bacterial content the incubation method outlined would have to be employed. This introduces an undesirable time factor which has yet to be overcome. Further work is planned in order to test the practical possibilities of this method and its accuracy.

SUMMARY

The direct count method for determining the bacterial content of fish muscle that is described could not be applied directly when there were less than approximately 1×10^5 organisms per gram of muscle. Samples containing fewer bacteria had to be incubated 24 hours at 15°C. before direct counts could be made. It was shown that there was absolutely no relation between bacterial counts obtained prior to and subsequent to such incubation, and the probable reasons for this were recorded. It was shown that such incubation procedure would probably give a more reliable index of the potential keeping quality of the fish than would a viable count made prior to incubation.

Direct counts usually, but not invariably, yielded somewhat higher results than did viable counts made on identical material, but the course of bacterial increase as measured by the two methods was very similar. The rise and fall of bacterial populations in fish muscle was in general distinguished by the same growth phases which are found in pure cultures of bacteria. Bacteria increased very much more slowly in whole fish than in excised fish muscle. In salmon muscle bacteria developed less rapidly than in halibut muscle.

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Biological and Oceanographical Conditions in Hudson Bay.

11. Polychaeta from Hudson Bay.

By E. and C. Berkeley
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ABSTRACT

Fifty-seven species of Polychaeta are recorded, of which forty-nine are well known northern species, six are species which have been recorded previously from North America, but not from the north coast and two are new species forming a new genus, *Pseudosabellides littoralis* and *lineata*.

INTRODUCTION

The last communication in this series appeared some nine years ago (Bell and MacFarlane 1933). At about the same time the Polychaeta collected during the four expeditions dispatched to Hudson bay under the auspices of the Biological Board of Canada in the years 1927-1930 were handed to us for determination, but we have been able to undertake the work only recently. Details of the dates, personnel, etc., of these four expeditions will be found in paper no. 9 of the series by V. D. Vladykov (1933).

RECORDS

We have identified fifty-seven species of Polychaeta in the material collected. With the exception of six of them (*Harmothoe imbricata*, *Lagisca rarispina*, *Nereis pelagica*, *Lumbrinereis fragilis*, *Cistenides granulata*, and *Spirorbis spirillum*), which have already been noted from Hudson bay and Hudson strait by Chamberlin (1920), all are new records for the region. All, except eight, are forms which have been fully and, in many cases, repeatedly described from northern Europe or Greenland by Scandinavian, British, and French authors and no more is now necessary than to record the extension of their distribution to Hudson bay. They are as follows:—

Harmothoe imbricata Linné
Eunoe nodosa Sars
Gattayana cirrosa (Pallas)
Lagisca rarispina Malmgren
Phyllodoce groenlandica Oersted
Phyllodoce mucosa Oersted
Capitella capitata (Fabricius)
Arenicola marina Linné
Maldane sarsi Malmgren
Nicomache minor Arwidsson
Axiothella catenata Malmgren
Praxillella affinis (Sars)

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Phyllodoce maculata (O. F. Müller)
Eteone longa (Fabricius)
Castalia fabricii Malmgren
Syllis hyalina Grube
Nereis pelagica Linné
Nereis zonata Malmgren
Nephthys ciliata O. F. Müller
Nephthys paradoxa Malmgren
Nephthys longosetosa Oersted
Ophryotrocha puerilis Claparède
Lumbrinereis fragilis (O. F. Müller)
Spio filicornis (O. F. Müller)
Polydora caeca (Oersted)
Polydora quadrilobata Jacobi
Polydora ciliata (Johnston)
Cirratulus cirratus (O. F. Müller)
Flabelligera affinis Sars
Brada villosa (Rathke)
Ophelia limacina (Rathke)
Ammochares fusiformis (Delle Chiaje)
Cistenides granulata Linné
Cistenides hyperborea Malmgren
Ampharete gracilis Malmgren
Ampharete arctica Malmgren
Melinna cristata (Sars)
Nicolea zostericola (Oersted)
Leaena abranchiata Malmgren
Pista maculata (Dalyell)
Terebellides stroemi Sars
Sabella crassicornis Sars
Chone infundibuliformis Kröyer
Euchone analis (Kröyer)
Dasychone infarcta (*Kröyer*)
Fabricia sabella (Ehrenberg)
Spirorbis spirillum (Linné)
Spirorbis granulatus (Linné)
Spirorbis pagenstecheri Quatrefages

Six of the species which are represented in the collections have been described previously from North America, but not from the north coast. A list of these, with notes of their previous records follows:—

Evarnella triannulata (Moore)	Known from south California (Moore 1910) and Vancouver island (Berkeley, E. and C. 1942).
Eunoe depressa Moore	Known from Alaska (Moore 1905).
Eulalia levicornuta Moore	Known from south California (Moore 1909) and Vancouver island (Berkeley, E. 1924).
Haploscoloplos kerguelensis (McIntosh)	Known from the west coast of North America as far north as Vancouver island (Berkeley, E. and C. 1941).
Tharyx acutus Webster and Benedict	Known from the coast of Maine (Webster and Benedict 1887).
Scionella estevanica Berkeley	Known from Vancouver island (Berkeley, E. and C. 1942).

The remaining two species are new to science. It has been found necessary to establish a new genus of Ampharetidae to contain them.

GENUS **PSEUDOSABELLIDES** g. n.

Definition. Like *Sabellides* Milne-Edwards (Malmgren and Hessle char. emend.) in having a trilobed prostomium, pinnate retractile tentacles, and four pairs of branchiae. Differs from that genus in having no trace of paleae and in having fourteen setigers, beginning on the fourth somite, and twelve uncinigerous tori, beginning on the sixth somite, in the thorax.

(*Note.* *Sabellides* has the paleae very reduced or absent and thirteen setigers, beginning on the fourth somite, and eleven uncinigerous tori, beginning on the sixth somite, in the thorax.)

Genotype. *Pseudosabellides littoralis* sp.n.

Pseudosabellides littoralis sp. n.

Average size 25 mm. long by 3 mm. wide. A well defined ventral groove extends over the last two thoracic and the first three abdominal somites and there is a sharp constriction between the third and fourth abdominal somites. Usually two simple eyes can be made out. There are eighteen to twenty abdominal somites with uncinigerous pinnules. The eight branchiae are smooth and subulate. Six of them form an unbroken row on the edge of a fold across the dorsum of the third somite, the other two are behind the second from either end of the row and originate near the base of the fold. The dorsal ramus of the parapodium in the thoracic region, and the setae it carries, are as Fauvel (1927) describes for *Sabellides octocirrata* Sars. The ventral ramus in the abdominal region is a rather broad lobe and it is surmounted by a short broad cirrus in the more posterior somites. This cirrus is very inconspicuous in the more anterior somites and never projects appreciably beyond the end of the pinnule. The shape of the uncinus is like that of *Sabellides borealis* Sars as figured by Malmgren (1865, pl. 20, fig. 47d). It has two rows of teeth, each row containing three or four members. The teeth alternate in the two rows so that all are seen as one row in a full profile view of the uncinus. There are two rather long anal cirri. The tubes are coated with coarse sand-particles and are very fragile.

Two specimens in this collection are from Port Burwell, Ungava; a third from Wakeham bay, Ungava. The species is common in the region of Nanaimo, Vancouver island. In all cases it has been collected above low tide mark.

The type is in the authors' collection.

Pseudosabellides lineata sp. n.

This is a long slender species. The largest specimen known is 15 mm. long and only 0.6 mm. wide. There are usually only fourteen to sixteen abdominal segments, but a few examples have as many as twenty. The branchiae are on the third somite and are arranged as in *Ps. littoralis* except that the posterior pair are nearer the median dorsal line. They are very slender, almost filiform. The dorsal ramus of the parapodium in the thoracic region and the setae it carries are much as in *Ps. littoralis*, but the ventral ramus in the abdominal region differs from that species in bearing a slender cirrus which extends beyond the pinnule. The shape of the uncinus is also different. It resembles that of *Sabellides octocirrata* Sars as figured by Fauvel (1927, fig. 81f). The tube is tough and fits the animal closely. It is coated with smooth mud in which some sand may be imbedded.

All the specimens known have been taken below low tide mark. Those in this collection were all dredged at Port Burwell, Ungava, in 10 to 15 fm. (18 to

27 m.). It has also been taken off the east coast of Vancouver island in 25 fm. (46 m.) and off the coast of Alaska in 8 to 12 fm. (15 to 22 m.). It was recorded from the Alaskan localities, erroneously, as *Sabellides octocirrata* Sars (Berkeley, E. and C. 1942).

The type is in the authors' collection.

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Bottom Fauna of Portions of the Cowichan River, B.C.

By Clarence Purvis Idyll
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(*Received for publication February 11, 1943*)

ABSTRACT

The yields per sq. m. computed as (1) av. wet wt. and (2) no. of organisms were: pool—36.91 g., 745; riffles—14.60 g., 1061; creek—8.25 g., 536. Vegetation is the most productive type of habitat, followed by mud, rubble, gravel bottom. The river may be designated as a "*Trichoptera* stream". No close correlation was found between no. of organisms in bottom fauna and no. in trout stomachs, thus suggesting discrimination among trout for food organisms.

This report summarizes the results obtained from studies made in a biological survey of the Cowichan river system on Vancouver island, British Columbia, by the Fisheries Research Board of Canada during the months of May to August, inclusive, of 1938, 1939 and 1940. The assistance and advice of Dr. W. A. Clemens, at that time Director of the Pacific Biological Station, and of various members of his staff are gratefully acknowledged.

DESCRIPTION OF THE RIVER

The Cowichan river is about 28 miles (45 km.) long, flowing in an eastward direction from Cowichan lake into Cowichan bay on the east coast of Vancouver island. In general, the width varies from 100 to 200 feet (30 to 60 metres). Just below the lake the river widens into the "Hatchery" pool, 150 yards (137 metres) in width, about 400 yards (365 metres) long and with a maximum depth of 30 feet (9 metres) at low water, and about 38 feet (11.5 metres) at average high water. The bottom of the pool consists, in the upstream section, of coarse gravel and sand, elsewhere of mud with growth of pond weeds (*Potamogeton*) in the shallower portions.

Below the pool there is a short rapid, and below this again a series of smaller pools, alternating with faster water. The bottom of the rapid consists mainly of rubble, with some sections of boulders and gravel, and a few patches of moss (*Fontinalis*), grasses and water forget-me-not (*Myosotis laxa*).

Oliver creek, an important spawning stream for salmon and trout, enters the river at the first riffles below the pool. It carries a small but persistent volume of water throughout the summer. The bottom consists of sand and gravel, with some rocks and rubble.

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METHODS

In the upper part of Cowichan river and in Oliver creek samples were taken at ten stations selected to represent various types of stream bottom. Most samples were taken with an Ekman dredge, but for the shallow water areas, e.g. station no. 5, a special sampler was designed, consisting of a rectangular wooden box, 18 in. (45 cm.) high, 16 in. (41 cm.) wide and 17 in. (43 cm.) long. Fine meshed galvanized wire cloth (12 meshes to the inch) covers the front and a net of fine marquisette (30 meshes to the inch) is attached at the back. The box is strengthened by triangular blocks set into the corners at the top. These blocks serve also as shelves on which to set the pan. Wooden handles on the sides facilitate the handling of the box.

To operate, the sampler is set firmly into the stream bottom and the material thus enclosed is scooped into deep iron pans such as are used to hold Ekman dredge samples. Organisms knocked loose as the bottom materials are being scooped up are swept into the net by the current. In quiet sections where no current exists a hand sieve captures such organisms.

This sampler works well in water up to 17 in. (43 cm.) in depth, even in quite fast current. Being on the same general principle as the frame sampler it is equally efficient in ordinary types of bottom, though not entirely satisfactory in rubble. Its larger size allows a wider selection of bottom area to be sampled, and permits the complete enclosure of more rocks. Stones and gravel may be banked around the sides, serving to prevent the escape of the water due to irregularity of the bottom. This obviates the need of a net behind the sampler, as is necessary with the square-foot box. The larger area enclosed by the new sampler allows more room to scoop out the bottom material, and while greater amounts of material are thus obtained, making picking over a longer task, fewer samples are necessary for accurate results.

In the laboratory the organisms, after being drained for one minute on blotting paper, were counted and weighed on a torsion balance to two places of decimals. In reporting weights, that of the shell of molluscs has been deducted. Caddisfly larvae were removed from their cases before being weighed. Weights may not be perfectly comparable for all samples since some were weighed alive (when possible) and others after having been preserved for varying lengths of time (up to several months), in eight to ten per cent formalin.

Although studies of stream bottom populations have long been recognized as imperfect, and have recently been seriously criticized (Mottley *et al.* 1938), the techniques employed in the present investigation serve the purposes for which they were designed. At least minimum figures for the abundance of bottom forms are obtained, and these have value for comparing one organism, or one type of organism, with another.

DATA AND RESULTS

Characteristics of Fauna in Different Habitats (tables I and III)

In the pool area—mud bottom, aquatic plants and organic detritus, slight to moderate current—the chief organisms were molluscs, aquatic insects and

TABLE I. List of sampling stations occupied (upper part of the river and Oliver creek), respective characteristics, and number of samples taken

Station number	Name of station	Bottom character	Depth of water (m.)	Current	No. samples taken
1	Net house....	mud; thick growth of *Potamogeton*	1–2	very slight	14
2	Mid pool.....	mud; sunken bark, leaves, etc.	7–9	slight	19
3	Gravel, pool..	gravel	0.5–1.5	moderate	18
4	Foot of pool..	mud; moderate growth of *Potamogeton*	2.5–3.5	moderate	16
5	Edge of pool..	mud; grasses and rushes, decaying vegetation	0.15–0.45	slight	18
6	Rubble riffle..	rubble	0.15–0.45	moderate to brisk	18
7	Gravel riffle ..	gravel	0.15–0.45	moderate	20
8	Weedy riffle ..	mud and gravel; thick growth of moss and other vegetation	0.10–0.20	brisk	6
9	Creek riffle...	coarse gravel to sand	0.10–0.25	brisk	13
10	Creek pool....	rubble and coarse gravel	0.20–0.35	slight	19

shrimps, with the first predominating. Midges were the most abundant insect, except on the gravel bar (station 3) where mayflies constituted almost 50% of the forms. In the shallow reaches of the pool edge (station 5) crustaceans, largely *Hyalella azteci*, were particularly abundant, forming 63.6% of the organisms found.

In the shallow rubble riffles (station 6), which form a large proportion of the river bottom, caddisflies predominated, 59.2%, with mayflies forming 11.9% and molluscs, 9.5%. Flatworms made up 7.6%.

On the gravel riffles (station 7), while caddisflies were again predominant, followed by mayflies, 10.2%, stoneflies appeared to the amount of 8.1% and molluscs represented only 1.5% of the total organisms found.

In the weedy riffle (station 8) blackflies constituted 80.5% of the forms dredged up. It is apparent that the sampling here was done before these insects had emerged. Mayflies, shrimps, midges, molluscs and caddisflies occurred in appreciable numbers.

In the creek habitat, little difference was noted between the shallow pools and the gravel riffles. Insects, chiefly mayflies, midges and caddisflies, were abundant; stoneflies and craneflies appeared in numbers but molluscs were rare and crustaceans absent.

PRODUCTION IN DIFFERENT HABITATS (TABLE II)

Considering the area as a whole, i.e., all stations combined, the average wet weight of organisms was 24.49 grams per sq. m. and the average number, 798 per sq. m. Thus the Cowichan river may be considered relatively poor in bottom forms, if gauged according to Needham and Hanson (1935) who designate the streams of the Sierra national forest as "poor in food organisms" since they

TABLE II. Summary of average weights and number of organisms at all stations

Station	Name of station	Av. wt. in g. per sq. m.	Average no. per sq. m.	Rank by weight	Rank by number
1	Net house	68.30	1789	1	2
2	Mid pool	60.11	399	2	7
3	Gravel, pool	1.35	404	10	5
4	Foot of pool	50.94	822	3	3
5	Edge of pool	3.89	321	9	9
6	Rubble, riffles	23.31	378	4	8
7	Gravel, riffles	7.22	285	7	10
8	Weeds, riffles	13.29	2521	5	1
9	Current, creek	4.72	676	8	4
10	Pool, creek	11.78	396	6	6
Average	Whole area	24.49	798		
Average	Pool	36.91	745		
Average	Riffles	14.60	1061		
Average	Creek	8.25	536		
Average	Stations 6 and 7	15.26	331		

yield only 1539 per sq. m. as compared with 3983 and 4090 per sq. m. in the streams of the Mono and Inyo (Smith and Needham 1935), and Shasta and Klamath national forests (Taft and Shapovalov 1935). The Cowichan river provided approximately one half of the standing crop of the "poor" streams of the Sierra forest. Surber (1936) shows his experimental stream to yield an average of 5.047 and 6.695 grams per square foot wet weight in two successive years. The Cowichan is much below this, yielding only 2.28 grams per square foot wet weight.

TABLE III. Percentage occurrence of and numbers of organisms eaten by

Organism	Station number									
	1	2	3	4	5	6	7	8	9	10
Caddisflies	0.9	2.8	6.8	5.0	1.7	59.2	60.2	2.2	19.2	23.5
Blackflies	....	....	....	0.2	....	0.2	1.3	80.5	7.0	....
Midges	29.9	19.9	12.3	11.8	1.7	4.6	1.5	2.8	28.0	36.0
Mayflies	0.6	1.3	49.2	3.3	2.6	11.9	10.2	5.8	29.4	22.0
Stoneflies	...	...	8.4	....	....	1.5	8.1	x	6.7	4.3
Dragonflies and damselflies	1.6	1.2	....	1.8	1.3	....	0.1	0.2	....	....
Aquatic beetles	x	0.5	2.1	0.1	0.3	x	5.4	0.2	0.4	0.6
Crustacea	14.9	28.4	8.4	29.4	63.6	4.1	3.6	5.1	....	....
Mollusca	45.3	32.8	2.1	33.0	17.7	9.5	1.5	0.5	0.3	0.2
Annelids	3.3	4.4	6.5	7.1	4.7	0.4	1.1	2.4	1.1	5.9
Flatworms	1.7	1.0	2.9	4.9	1.8	7.6	2.3	x	0.9	....
Leeches	0.6	5.6	1.0	2.2	2.8	0.4	3.3	x	....	....
Craneflies	....	0.7	....	0.1	0.3	....	1.1	x	5.4	6.7
Miscellaneous	3.0	1.0	....	1.0	0.5	x	....	x	1.0	1.0

x = present in insignificant percentage.

With reference to the various habitats within the river, the pool, excluding the gravel bar and the shallow margins, provided an average of 60.11 grams wet weight per square metre, as compared to 23.31 for the rubble riffle, 13.29 for the weedy and but 7.22 for the gravel. In numbers of organisms per square metre, the weed riffle showed greatest abundance because of the coincidence of the sampling with a profusion of blackfly larvae. The next most productive was the upper part of the pool (station 1) where the mud bottom, thick growth of *Potamogeton* and moderate depth of water with very slight flow, provided excellent conditions. The striking discrepancy between average weight and numbers of organisms in station 2 is due to the presence of numerous large clams, *Anodonta* sp.?, which caused the weight to be disproportionately high. Oliver creek was well below the other areas in both weight and number of organisms. Stations 6 and 7, rubble and gravel riffles, represent the type of bottom of which the greatest area of the river is composed and the average yield (15.27 g. wet weight per sq. m.) is shown to be below the general average for all stations (24.49 g. per sq. m.).

In general, vegetation is the most productive type of habitat, followed by mud, rubble and gravel, in that order. This is more or less in accordance with the findings of the majority of workers (Percival and Whitehead 1929); P. R. Needham 1934, 1938), except that mud is sometimes stated to have been less productive than rubble (Moore *et al.* 1927).

RELATION OF BOTTOM FAUNA TO FISH FOOD

The portions of the Cowichan river studied in this investigation support an important number of fish. The Hatchery pool and the riffles below are favourite

each organism at each station
561 trout caught in the river

Average %	Rank	Number eaten by 561 river trout	Percentage of organisms eaten	Rank by no. eaten	Av. % occur.	Rank by occur.
18.17	1	3079	11.4	2	56.7	1
8.82	6	21398	79.3	1	20.2	4
14.85	3	1108	4.1	3	28.3	3
13.63	5	441	1.5	5	31.0	2
2.90	8	462	1.5	4	14.9	5
0.62	13	35	0.1	7	5.6	7
0.96	12	2	x	..	1.3	..
15.75	2	7	x	..	2.3	..
14.29	4	430	1.5	6	6.4	6
3.69	7	....	....	..	....	..
2.31	9	....	....	..	....	..
1.59	10	....	....	..	....	..
1.43	11	....	....	..	....	..
0.80	..	23	x	..	2.7	..

places for angling, and Oliver creek is a spawning stream of considerable importance for both salmon and trout. No attempt has been made to separate the data for the food of pool, riffles and creek trout for there is no evidence to indicate that trout feed exclusively in any one of these areas at all times. On the contrary, it is probable that considerable movement takes place between pool and riffles and between creek and river.

The average percentages of the major groups of bottom organisms for all stations (table III) show no exact correlation with the numbers and percentages of the chief organisms (table III) eaten by 561 rainbow, cutthroat and brown trout of all sizes, caught by various types of gear in approximately the same area where the bottom samples are taken. The closest relationship occurs in the case of caddisflies, which are the most numerous organisms present, occur oftenest in the stomachs, and are eaten in the second largest numbers. Midges rank third in all three categories. Mayflies rank fifth in numbers present in the fauna and numbers eaten, but second in percentage occurrence in the stomachs. The other groups show greater disparity, in varying degrees, the greatest occurring in the fresh-water shrimps, which rank as the second most numerous organism in the fauna but which are not used as food by the trout to any significant extent. Molluscs also appear not to be used in proportion to their incidence in the population.

There are probably three main reasons for lack of correlation between numbers of organisms present and numbers used as trout food. The first is the fact that percentage occurrence does not actually represent the relative numbers for the whole region, since the areas of the various bottom habitats are not taken into account. The second is the fact that percentage occurrence does not necessarily mean numbers available as food. This probably accounts largely for the small importance of such groups as the annelids, flatworms and *Anodonta* in the fish food. Groups such as caddisflies and mayflies, which are both numerous and readily available as food due to size and habits, will naturally make up a large proportion of the food. The third factor influencing the importance of an organism as food for trout is possible selectivity. In a previous report (Idyll 1942) a definite difference in the selectivity of the three species of trout (rainbow, cutthroat and brown) in the Cowichan river system was indicated, as revealed by differences in the food of these three species under similar feeding opportunities. The present results tend to confirm such a conclusion.

There has been much controversy on this question and two opinions are quoted as illustrating the opposite viewpoints. Neill (1938), in a study of brown trout food in relation to the bottom fauna in England, concludes that "the trout feeds on the whole range of animals present in whatever type of habitat it finds itself, to an extent dependent on their degree of accessibility and the extent of their representation in the fauna. This is sufficient to account for the nature of its stomach contents without invoking discrimination on the part of the fish." In contrast, Allen (1938), reporting the food of brown trout from Windermere, England, states that "Percentages which the most important food animals make up of the food are probably very much greater than are their percentages in the

fauna at the same time, i.e. the fish are performing a definite selection in their food." A great deal of further research will be necessary before a final decision can be made.

CONCLUSIONS

(1) The portions of the Cowichan river studied in this investigation are poor in bottom fauna.

(2) The Hatchery pool is more productive than the riffles below on the basis of weight of organisms (36.91 vs. 14.60 g. per sq. m.), but less productive on the basis of numbers (745 vs. 1061 organisms per sq. m.). Oliver creek is less productive (8.25 g. and 536 organisms per sq. m.) than the other two areas.

(3) Vegetation is the most productive type of habitat in this river, followed by mud, rubble and gravel bottom, in that order.

(4) The Cowichan river may be designated as a "*Trichoptera* stream", since this group is distinctly dominant among the bottom fauna.

(5) There is no close correlation between numbers of organisms present in the bottom fauna and the numbers taken by the trout for food. This may possibly suggest discrimination on the part of the trout in relation to food taken.

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Sex, Gonad Development and Seasonal Gonad Changes in *Paphia staminea* Conrad

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(*Prepared for publication by Ferris Neave and R. E. Foerster in the author's absence on Active Service*)

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ABSTRACT

In the little-neck clam, *Paphia staminea*, as occurring in British Columbia waters, the first sign of the gonad was found in individuals slightly over 1 mm. in length. Sex differentiation occurs at lengths of 15 to 30 mm. or in 2nd or 3rd year. Maturity is achieved at 22 to 35 mm. *P. staminea* is not protandric. Seasonal gonad changes are: by end of October all active spawning over; by December to January tubules of ovary filled with follicle cells; active growth of sex cells reaches peak in March; first spawning in April and by September most clams spawned out. Male spawning cycle parallels that of female, with certain time lag apparent. Some males may remain in spawned-out state during most of winter.

Sex in molluscs has been studied by scientists since the end of the 17th century. Most of the workers (Amemiya 1929, Belding 1912, Burkenroad 1931, Coe 1932, 1936b, Orton 1927, etc.) used various species of the genus *Ostrea* as the subject of their studies. More recently, however, careful investigation has been made of the sexuality in species of various other genera of pelecypods, i.e., *Teredo* (Coe 1936a), *Venus* (Loosanoff 1936), and *Mya* (Coe and Turner 1938). A knowledge of the sexuality of a molluscan species is necessary for an adequate understanding of its life history and the relation of that life history to the problems of regulation and conservation.

In pelecypods and gastropods sex changes often occur as a result of age, rapidity of growth, or differences in environment. Some species undergo a prolonged and complex series, others may have only one, while in some there may be none at all. Many species exhibit partial or complete protandry, or proterogyny.

ACKNOWLEDGMENTS

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PART I. SEX DIFFERENTIATION, GONAD DEVELOPMENT AND GAMETOGENESIS

Materials and Methods

Samples of small clams were collected by means of a fine sand screen from a beach on the eastern point of Woods island in Ladysmith harbour. Other samples were obtained from oyster cultch. The clams varied in size from 1 to 30 mm. in length, taking the greatest antero-posterior measurement. Serial sections were made of the smaller individuals but from the larger individuals a small section of the gonad, as near as possible from the same location on each clam, was removed and several sections prepared. Approximately 800 slides were utilized in this study.

Standard methods of histology were followed. Bouin's picro-acetic-formol of the 75-5-25 proportion was the fixative. Both Ehrlich's acid haematoxylin and Heidenhain's iron haematoxylin with ferric alum mordant were used; also, in several instances, a saturated solution of picric acid for differentiation after iron haematoxylin. Excellent differentiation was obtained, the cytoplasm being left quite colourless and the nuclei retaining the stain well.

The paraffin technique, with cedar oil and xylol as clearing agents and alcohol for dehydrating, was adopted initially, but, after trial, the dioxan technique for dehydrating was found to be the most satisfactory both from the standpoint of quality of results and saving in time and detail. It was used exclusively throughout the latter part of the investigation.

Where serial sections were to be made of small whole clams, the shells were partly dissolved off by the action of the acid fixative and whatever remained was removed by dissection. Sections were cut 3 to 10μ thick. Aqueous yellowish eosin was used as a counter-stain in some cases.

Size and Age

Since *Paphia* has a long spawning season, extending from May to the end of September or even October, considerable variation exists in the size of young clams of the year as they enter the winter, or as they begin growth in the following spring. Indeed, specimens of the same year class have been found to vary between 1 and 10 mm. at the end of their first winter, and in the following summer this variation is extended even further owing to differential growth rate. Accurate age determination, therefore, is difficult.

Development of the Primary Gonad

The primary gonad begins as two primordia, one on each side of and immediately ventral to the pericardial cavity in the posterior part of the body (fig. 1, A). Soon, however, the two primordia meet and fuse centrally for a short distance.

At this time the dorsal aspect of a frontal section appears in the shape of the letter H. Also at this time the two main tubes descend somewhat into the musculature of the body, as far as the cerebro-visceral connectives to which they become attached. A cross-section of the gonad has the appearance of an inverted U.

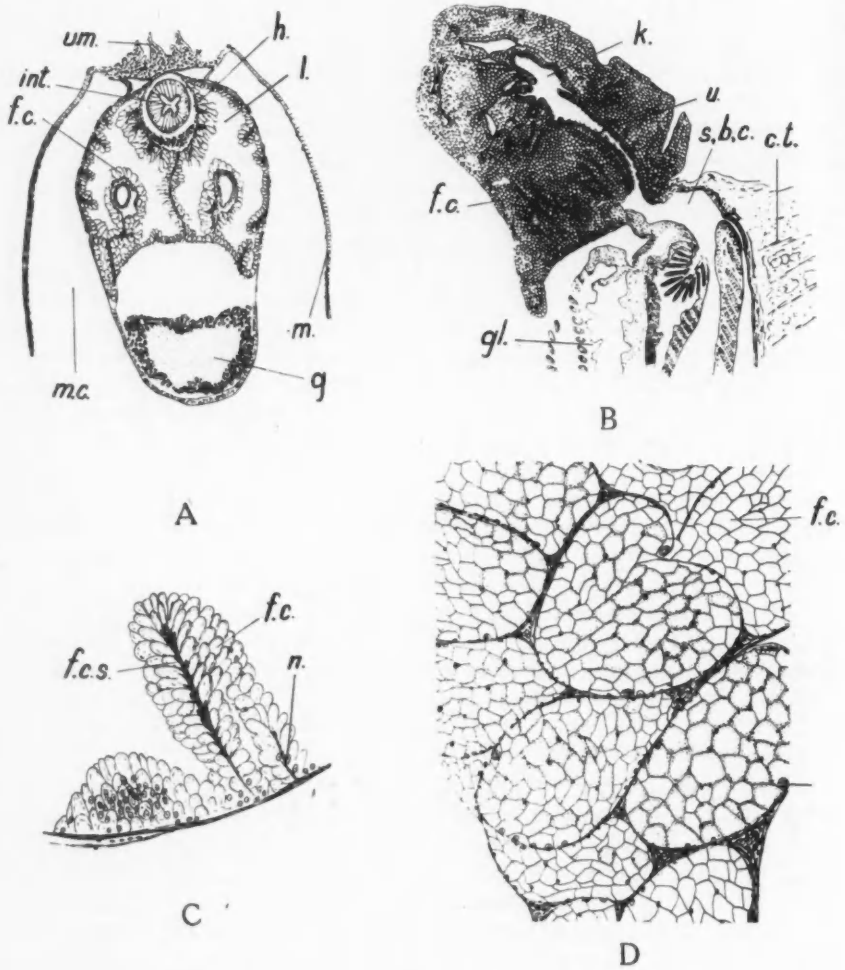

FIGURE 1. Young *Paphia*. A: cross-section of clam 4 mm. long showing primary gonad. B: section of gonad of clam 1.24 cm. long, June. C: enlarged section of gonad of clam 1.26 cm. long. D: section of gonad of clam 2 cm. long. (ct.—connective tissue, f.c.—follicle cells, f.c.s.—follicle cell stalk, g.—ganglion, gl.—gill, h.—heart, int.—intestine, k.—kidney, l.—lumen, m.—mantle, m.c.—mantle cavity, n.—nucleus, p.g.c.—primary germ cells, s.b.c.—suprabranchial chamber, u.—ureter, um.—umbo).

The same general position of the primary gonad is maintained throughout its development and it is only after sexual differentiation that the gonad proliferates to surround completely the foot and visceral mass.

The first indications of the gonad were found in clams slightly over a millimetre in length. The primordia at this time consist of a tubular structure of germinal epithelium upon which is laid a single layer of vacuolated follicle cells. The centre of the tubule is vacant. In the early stages of development of the gonad and kidney, the two organs are closely allied (fig. 1, B).

In clams 3 mm. in length, the gonad is distributed vertically between the upper and lower limits of the posterior adductor muscle. Horizontally it extends forward from the visceral ganglia, just in front of the posterior adductor muscle, for a distance of about one-third the length of the body.

Gradual extension of the gonad is accomplished by the growth and spread of the germinal epithelium forward and ventrally. Meanwhile the interior of the gonad is becoming more and more compact by the evagination and growth of parts of the germinal epithelium to form what might be called follicle cell "stalks", for the evagination of the epithelium carries with it the layer of follicle cells (fig. 1, C). At the same time the follicle cells are multiplying and a number of layers are formed (fig. 3, A). Formation of profusely branching tubules is finally brought about by growth and fusion of the follicle cell "stalks". This is accomplished when the animals reach a length of between 15 and 30 mm. (fig. 3, B), the size depending upon environment, inherent tendencies and time of setting. It is not until complete formation of the tubules that sexual differentiation into the male and female phases takes place. At this time the clams are in their second or third years.

The follicle cells are apparently nutritive in function for their disappearance coincides with the development and growth of the sex cells. The delicate cell wall of the follicle cell encloses a thin layer of cytoplasm which is often difficult to observe. The central portion of the cell is occupied by a large vacuole and the comparatively small nucleus is imbedded in the peripheral cytoplasm. The cells differ much in size and shape and may reach a diameter of 30μ. As gametogenesis proceeds the follicle cells are gradually replaced by the developing sex cells. After spawning there is a redevelopment of the follicle cells in the gonad tubules.

Sexual Differentiation

It was found that *P. staminea* is not protandric. As soon as the gonad tubules are fully formed, indifferent gonia, the sex of which cannot be determined, arise on the epithelium. Very soon, however, for the process is almost continuous, the indifferent gonia become differentiated into ovogonia (fig. 2, A) or spermatogonia. The former are slightly larger than the latter which measure, on the average, 8μ. The coarse, granular cytoplasm of the ovogonia is better developed than the corresponding part of the male cells. The nucleus of the spermatogonium is quite large and nearly fills the cell.

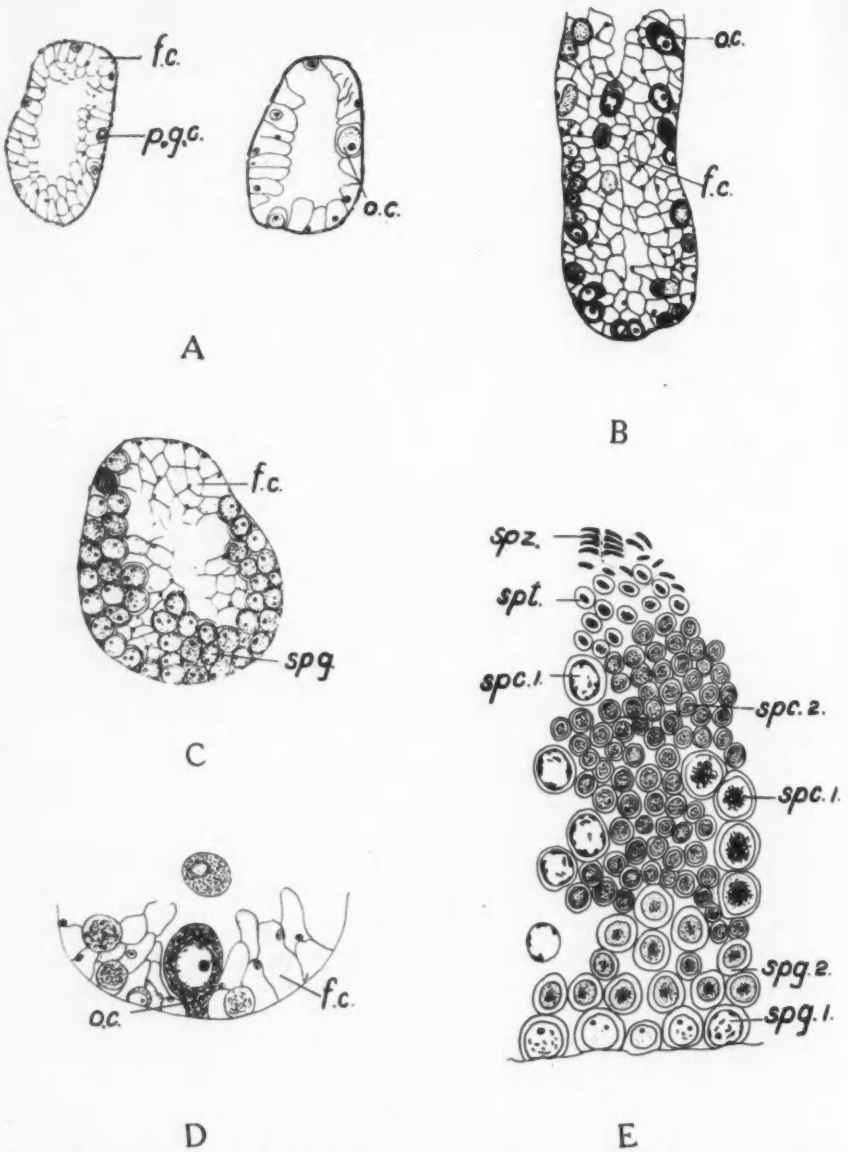

FIGURE 2. *Paphia*. A: sections of gonad tubules showing differentiation of female sex cells. B: section of more advanced tubule. C: section of gonad of male clam 2.5 cm. long, showing proliferation of gonia and disintegration of follicle cells. D: enlarged portion of ovary. E: semi-diagrammatic representation of spermatogenesis. (f.c.—follicle cells, o.c.—ovocyte, p.g.c.—primary germ cell, s.p.c. 1 and 2—primary and secondary spermatocytes, s.p.g. 1 and 2—primary and secondary spermatogonia, spt.—spermatid, spz.—spermatozoon).

Since the size of individuals at completion of the development of the primary gonad varies considerably, so does the size at sexual maturity, which is between 22 mm. and 35 mm. This conclusion supports that of Fraser and Smith (1928).

Ovogenesis

After sexual differentiation, or after the hibernation period in mid-winter in the case of adult clams, the ovocytes begin to grow and enlarge (fig. 2, B, D; fig. 3, C), apparently at the expense of the follicle cells, until they are attached to the walls of the follicles only by a slender stalk. When a size of between 55 and 63μ is reached, they drop off into the lumen of the gonad tubules and are ready to be extruded. By the time the ova are ripe and fill the tubules, few or no follicle cells remain. Besides the numerous ripe ova there are many small ovocytes and gonia still attached to the germinal epithelium.

Spermatogenesis

Owing to the extremely small size of the male reproductive cells of *P. staminea* the exact details were not distinguished. However, the process in general can be outlined. After sexual differentiation, or from March to May in the case of mature clams, the spermatogonia in the germinal epithelia of the gonad tubules grow and divide until a layer of spermatogonia several cells deep is formed (fig. 3, D). The primary spermatogonia are usually found in close contact with the germinal epithelium, although some may be found some distance from it. They measure approximately 8μ. The nucleus almost fills the cell and is somewhat oval in shape (fig. 2, C). The chromatin is dispersed and frequently two nucleoli are found. Often superimposed on the primary spermatogonia are gonia of the second order, whose round nuclei measure about 4μ in diameter (fig. 2, E). The secondary spermatogonia are often found dispersed some distance towards the centre of the lumen, for they are not always arranged in the order of their development, and may be found in almost any position in relation to each other, but definite layers of sex cells in regular order do occur. The nucleoli of the secondary spermatogonia usually occur close to the nuclear membrane.

Further in towards the centre of the tubules are groups of primary spermatocytes evolved by the division of the secondary spermatogonia. The primary spermatocytes are slightly larger than the secondary spermatogonia. As mentioned above, details of the transformation and division of the primary spermatocytes were not observed. The secondary spermatocytes are usually clustered together in large numbers. Their nuclei measure 2μ in diameter. The transformation of the secondary spermatocyte into the spermatid takes place after the last division of the spermatocyte by concentration of the chromatin of the nucleus into a solid heavily staining mass. The latter then begins to elongate and to assume the form of the mature spermatozoan head, 6μ in length, conical in shape and slightly curved. Excepting a single layer around the germinal epithelium the follicle cells are gradually crowded out and are replaced by the sex cells which finally become spermatozoa and align themselves in various patterns, heads together and tails together, in the lumina of the spermaries.

PART II. SEASONAL GONAD CHANGES

METHODS

Collections of adult clams were made as often as possible throughout the year from 1937 to 1940. During 1937, all samples were taken from one beach on Woods island in Ladysmith harbour. Subsequent collections were taken at

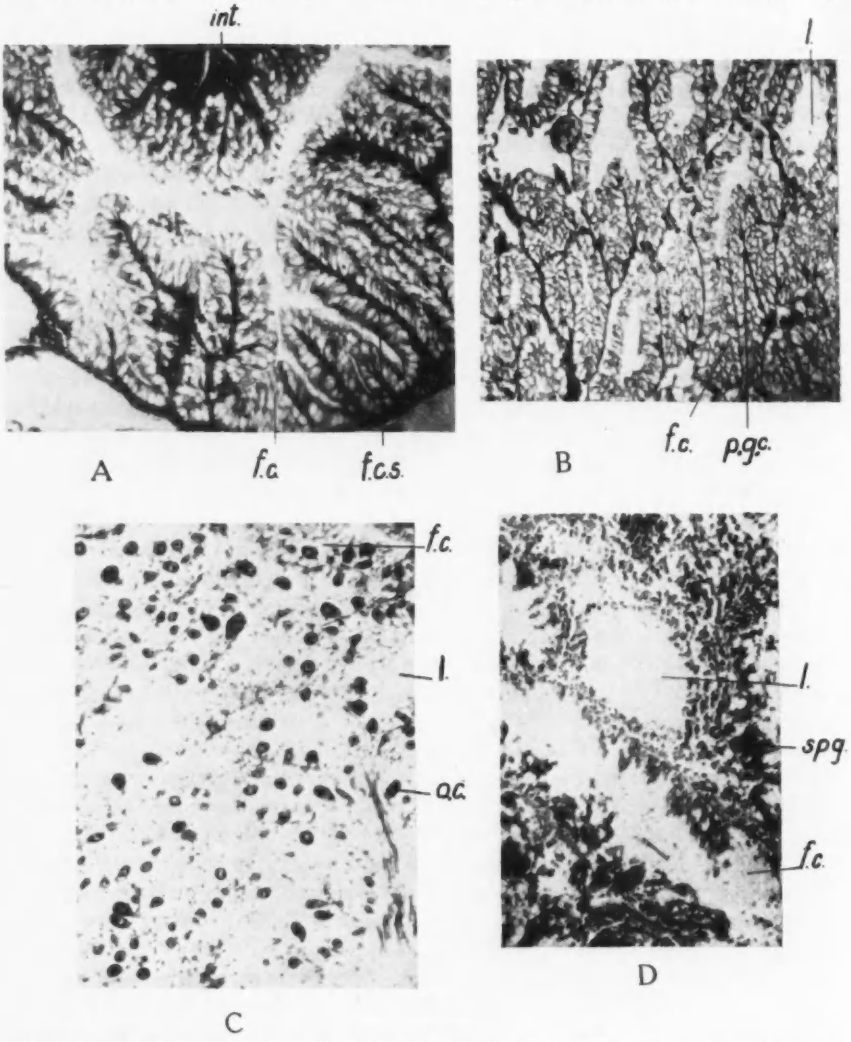

FIGURE 3. *Paphia*. A: section of gonad of clam 1.26 cm. long showing proliferation of the follicle cells, ×77. B: completed tubules in gonad of clam 2.64 cm. long, ×77. C: small new ovocytes in gonad of adult 4.16 cm. long, December, ×77. D: same as fig. 2c, ×77. (f.c.—follicle cell, f.c.s.—follicle cell stalk, int.—intestine, l.—lumen, o.c.—ovocyte, p.g.c.—primary germ cell, spg.—spermatogonia).

places wherever the author happened to be at the time of low tide and consequently the later samples are from a considerable number of beaches between Sidney and Alert bay on the east coast of Vancouver island. The greater part, however, came from the beach in front of the Pacific Biological Station at Departure bay.

The clams were opened by scalpel and a block of reproductive tissue, approximately 5 mm. square, taken from the body of the clam. The dissection was made as nearly as possible in the same region of the body, that is, the approximate centre of the right or left side of the animal.

The tissue was fixed in Bouin's picro-acetic-formol solution for approximately 24 hours. Standard histological methods were followed.

The preparation of sections by the freezing technique, in order to shorten the time, was successfully attempted.

Sections of gonad tissue from over 1,200 clams were prepared and form the basis of the present study.

Females

October is the last month of the year in which spawning of *P. staminea* takes place, and this commencement of a new sexual cycle appears to be a logical place to begin a discussion of the seasonal gonad changes.

By the end of October active spawning is over and all ovaries show a completely or partially spawned-out appearance. Even after complete spawning a considerable number of "residual" ova are usually left in the tubules of the ovary. In this discussion the term "ova" refers to ovarian eggs, or more specifically, primary ovocytes, for it is not until extrusion and fertilization of these latter that reduction division occurs. These residual ova may be extruded later, or may undergo cytolysis, or may even be maintained in the ovaries until the following year to be spawned with the next crop of eggs, for many of these morphologically mature ova are found in the lumina of the ovary surrounded by masses of developing ovogonia and ovocytes. Usually also, along with the residual ripe ova are a considerable number of young primary ovocytes.

After spawning, the walls of the tubules, consisting of the fine germinal epithelium studded with small primary ovogonia, are wrinkled and collapsed, with the lumina empty except for whatever few residual ova remain. Soon after spawning, however, proliferation of follicle cells begins, accompanied by slow development of primary gonia, the latter ceasing when a diameter of 10-15μ is reached (fig. 4, C). Growth of the follicle cells continues until the tubules are full or nearly so. This process is usually completed by December or January. Except for a short growth period immediately after spawning, the sex cells lie more or less dormant and show little activity until the end of December and sometimes even until well on into February. At that time, even though the temperatures are quite low, active growth begins (fig. 4, B) and is at its peak in March. During January and February considerable variation exists in the degree of ovogenic development in the various individuals. Follicle cells fill the lumina of the ovaries in all females except some which are already ripe (fig. 4, D) and whose ovaries are filled with mature ova. Gonia of various sizes and order and, in some,

ovocytes in all stages, line the epithelial walls of the lumina and are gradually replacing the peripheral follicle cells. By April the number of unripe females is relatively small and by the end of May the appearance of a ripening female is rare.

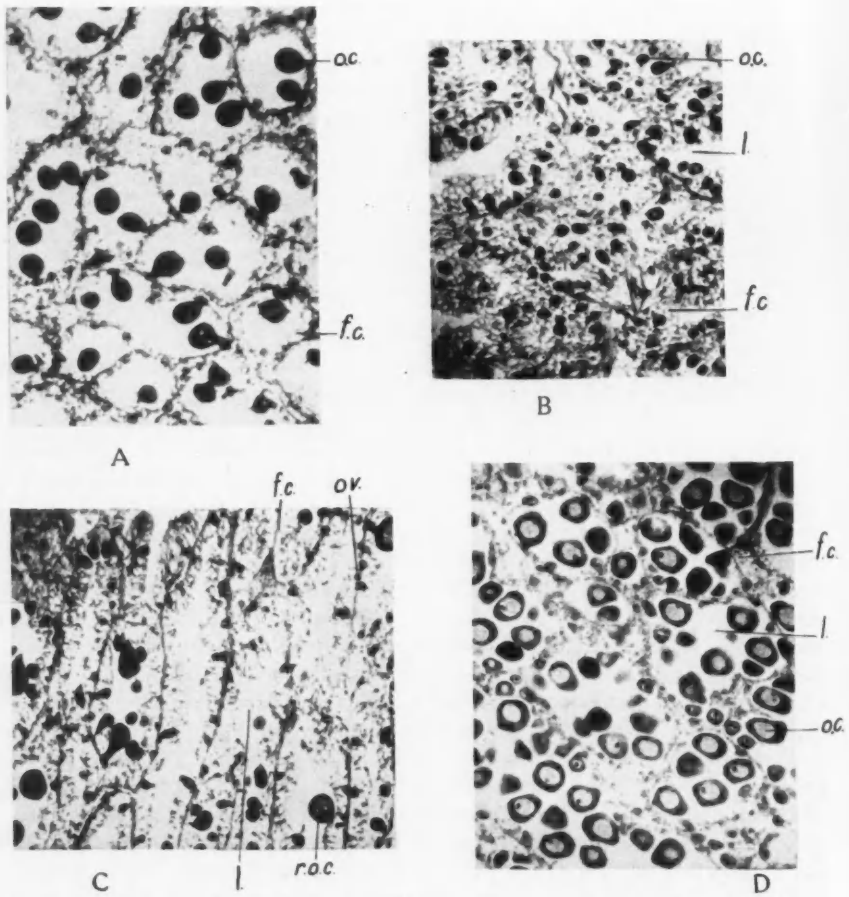

FIGURE 4. *Paphia*. A: relict ova in ovary of clam 3.56 cm. long after partial spawning, August, ×70. B: developing ovocytes in ovary of clam, January, ×70. C: residual ova and proliferation of new follicle cells in ovary of adult, November, ×70. D: ripe ovary of adult, with few follicle cells, ×70. (f.c.—follicle cell, l.—lumen, o.c.—ovocyte, ov.—ovogonium, r.o.c.—residual ovocyte).

April is the first month in which spawning occurs to any great extent, as evidenced by sections of clams with a spawned appearance and by the appearance of *Paphia* larvae in the plankton at both Ladysmith and Departure bay. However, a very small proportion of the clams which were sectioned gave indication

that isolated individuals may have spawned earlier. From April onward female clams in a spawned-out condition, partially spawned (fig. 4, A) or recovering, as indicated by follicle cell growth, exist in nearly equal proportions with a small number showing ovogenesis in the early stages.

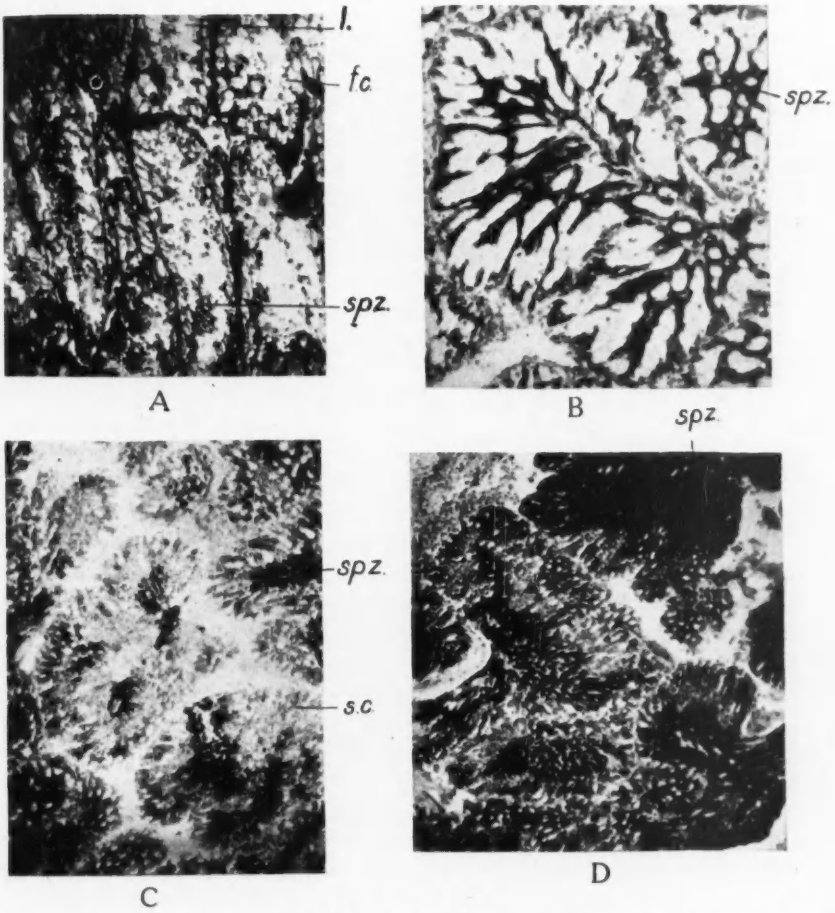

FIGURE 5. *Paphia.* A: gonad of adult clam just after spawning, August, showing collapsed tubules. B: spermary of poorly nourished clam, March, ×77. C: spermary of clam 3.0 cm. long showing developing sex cells. D: spermary of clam 5.38 cm. long, March, showing spermatogenesis almost completed, ×77. (f.c.—follicle cell, l.—lumen, s.c.—sex cell, spz.—spermatozoa).

By the beginning of September examination of slides shows the majority of the clams have spawned. Not all have done so, however, as indicated by the fact that larvae in considerable numbers are found even in early October.

It is not known whether *P. staminea* spawns more than once in a single summer, but it may be the case with a few individuals which spawn very early in the spring of the year. Indeed, Edmondson (1923) indicates that there may be two spawnings and such is the case with *P. philippinarum*, a closely related Japanese species.

Males

The male reproductive cycle follows that of the female fairly closely, although there seems to be a time lag of something more than a month. The recuperative period of the males is longer than that of the females and the former go through the fall and most of the winter in much the same state as they were immediately after spawning. Considerable numbers of morphologically mature males may be found in each month of the year.

Immediately after spawning, the tubules contain a number of spermatozoa held loosely in the centres of the lumina (fig. 5, A). The germinal epithelium containing a few inert gonia is collapsed. This appearance may be retained until January or February when proliferation of both follicle cells and spermatogonia begins almost simultaneously (fig. 5, C). The residual spermatozoa are retained until this time, but their ultimate fate is unknown. The follicle cells increase rapidly and soon fill most of the lumen. After the first crop of gametes has been produced the follicle cells in male animals are not able to fill completely the lumina of the tubules, differing in this respect from the females. In poorly nourished individuals (fig. 5, B), where the process can be observed most easily, groups of spermatogonia appear at intervals along the germinal epithelium. Superimposed on these are groups of secondary spermatogonia. Above these again are spermatocytes of both orders, and centrally are spermatids undergoing spermeoteleosis. First the centres and finally all of the lumina are filled with parallel columns of spermatozoa with the sperm heads ranged alongside each other and the tails intertwined in the clear space between the columns or rows of sperm heads. Well-nourished individuals show the tubules tightly packed and distended with reproductive cells in all stages of development, and arranged more or less as indicated above.

Spermatogenic activity reaches a peak between the end of March and early May (fig. 5, D). By the end of the latter month very few spermatogonia or spermatocytes remain, and the tubules are packed solidly with sperms. While spawning in the female may extend over a period of time, the male spawning is apparently completed at one time.

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Relation Between Moult Cycle and Phosphorus Content of Blood and Muscle in Lobster

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ABSTRACT

The concentration of inorganic and total phosphorus in lobster blood and muscle was determined. A wide variation in the phosphorus content of the blood was found, being highest immediately preceding the moult and lowest following it. The total phosphorus content of the muscle was relatively constant throughout the moult cycle, with an average concentration of 0.28 per cent, of which about 58 per cent was inorganic phosphorus.

INTRODUCTION

During an investigation of the causes of the formation of magnesium ammonium phosphate crystals in canned lobster, a brief study was made to determine to what extent, if any, the concentration of inorganic and total phosphorus in the muscle and blood of the American lobster, *Homarus americanus*, is influenced by ecdysis. The analyses were made during (1) the pre-moult period (lobsters nearing ecdysis, shell somewhat brittle, dark and dull in colour, and the bright pigment of the new carapace visible underneath the old; (2) the post-moult period (new shell not yet hard); and (3) the inter-moult period (shell hard and bright).

METHODS

Phosphorus was determined colorimetrically as inorganic phosphates by an adaptation of the method of Dyer and Wrenshall (1938), and of Smith *et al.* (1939). This is a modification of the method of Truog and Meyer (1929). The concentration was obtained by reference to a calibration curve established by using solutions of known phosphate concentration. For inorganic phosphates, protein was precipitated with trichloracetic acid and the filtrate used. This method of estimation possibly admits of a small increase in inorganic phosphates, due to the breakdown of labile organic phosphorus compounds. Rapid estimations should, however, have reduced this to a minimum. In total phosphorus determinations, organic matter was destroyed by digestion with perchloric and nitric acids. Organic phosphorus was derived by difference. In the estimation of total phosphorus in the muscle, approximately two-gram samples of flexor muscle were hydrolysed in a 70 ml. pyrex test tube with 6 ml. of perchloric acid. Heating was carried out on a specially-designed electric heater, and was continued until the muscle tissue had been destroyed and the resultant solution was

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dark brown to black in colour. The solution was then allowed to cool, 2 ml. of concentrated nitric acid were added, and again heated, cautiously at first, until digestion was complete and the solution colourless or nearly so. It was then made to volume in a 500 ml. volumetric flask, and an aliquot subsequently used for phosphate estimation. In the hydrolysis of whole blood and serum it was found advantageous and less time-consuming to add one ml. of concentrated perchloric acid and 0.5 ml. of concentrated nitric to 2 ml. samples, and to omit the preliminary heating of the samples with perchloric acid. Complete digestion of blood or serum was generally effected in 10 to 15 minutes. Digestion of muscle required approximately 30 minutes. Because of the explosive nature of a mixture of perchloric and nitric acids caution should be exercised in heating the solution after the addition of the latter.

The above method was found to be very satisfactory for the estimation of phosphorus in biological material, duplicate analyses varying less than five per cent. In order to determine the accuracy of the method, a number of phosphorus determinations were made on samples of blood and muscle, followed by redeterminations after the addition of known amounts of phosphate. Complete recovery was obtained.

Blood was obtained by breaking a cheliped at the basipodite or coxopodite. Duplicate analyses were made where possible. On two occasions the carapace in the region of the heart was removed and samples taken directly from the heart with the aid of a hypodermic needle. The phosphorus content of the samples thus obtained was identical with duplicates obtained from the great claws. Removal of blood from the heart was therefore discontinued. The clear fluid remaining after the clotting of the blood, which takes place very rapidly, was regarded as the serum. To estimate inorganic phosphorus in the blood, 2 ml. of blood were mixed with 8 ml. of 10 per cent trichloracetic acid, 5 ml. of the filtrate were diluted to 50 ml., and a suitable aliquot used for the estimation of phosphorus.

Analyses of inorganic and total phosphorus in the muscle were consistently made on the flexor muscle, the right muscle being used for one analysis, the left for the other. Ten per cent trichloracetic acid was again used as the protein precipitant in the estimation of inorganic phosphorus in the muscle.

RESULTS OF ANALYSES

Inter-moult

It will be seen (table I) that the total phosphorus content of whole blood was relatively constant during the inter-moult period, and that there was no apparent difference between that of the male and female. In contrast, the values for total phosphorus in serum showed a variation of well over 100 per cent. This variation cannot be attributed to faulty analytical methods, since duplicate analyses varied within 5 per cent. However, it was seldom possible to make duplicate determinations of inorganic phosphorus in whole blood because of the rapidity with which lobster blood clots. It would appear that there is no correlation between inorganic phosphorus content of whole blood, total phos-

phorus in serum, and total phosphorus in whole blood during the inter-moult period.

TABLE I. Phosphorus in lobster blood and muscle during the inter-moult period, expressed as grams P per 100 millilitres of blood or 100 grams of muscle tissue.

Sex	Serum	Whole blood		Muscle	
	Total P	Inorganic P	Total P	Inorganic P	Total P
Male	0.0029		0.0126	0.16	0.30
Male	0.0024		0.0132		
Male	0.0025	0.0017	0.0130		
Male	0.0063	0.0030	0.0131	0.14	0.26
Female	0.0042		0.0137		
Female	0.0090	0.0064	0.0138	0.13	0.26
Female	0.0056		0.0121	0.14	0.27
Female	0.0064	0.0021	0.0107	0.17	0.27

In the muscle, only a limited degree of variation was evident in the concentration of inorganic and total phosphorus (and therefore in the organic phosphorus content, obtained by difference) during the inter-moult period. Results of additional analyses of muscle made during this period verified the analyses given in table I, and indicated that the concentration of inorganic phosphorus varied from 0.13 to 0.16 per cent, and that of total phosphorus from 0.26 to 0.30 per cent. Inorganic phosphorus comprised on an average about 55 per cent of the total.

PRE-MOULT

Marked variation was evident (table II) in the phosphorus content of the blood and blood fractions during the pre-moult period. There was also a decided increase in the concentration of phosphorus, in many cases being more than double that of the inter-moult period. On an average the concentration

TABLE II. Phosphorus in lobster blood and muscle during the pre-moult period, expressed as grams P per 100 millilitres of blood or 100 grams of muscle tissue.

Explanatory	Sex	Serum	Whole blood		Muscle	
		Total P	Inorganic P	Total P	Inorganic P	Total P
Early pre-moult.....	Female	0.0089	0.0043	0.0141	0.16	0.295
Intermediate pre-moult	Female	0.0140	0.0065	0.0179	0.185	0.285
Intermediate pre-moult	Male		0.0083	0.0203	0.16	0.31
Intermediate pre-moult	Male	0.0109	0.0064	0.0205	0.16	0.31
Intermediate pre-moult	Male	0.0105	0.0062	0.0183	0.18	0.27
Advanced pre-moult...	Male	0.0204	0.0130	0.0272		

of inorganic and total phosphorus in the flexor muscle was little higher than those during the inter-moult period, but the variation was not of an order to be significant.

POST-MOULT

The values for inorganic and total phosphorus in the muscle during the post-moult period (table III) were comparable to those of the pre-moult period. There was, however, a sharp drop in the phosphorus content of the blood fractions. The values for total phosphorus in the blood were approximately half those for the inter-moult period, and one-third those for the pre-moult period. The greatest proportionate drop was in the inorganic phosphorus content of the blood, while there was a smaller variation in total phosphorus in the serum than at any other period.

TABLE III. Phosphorus in lobster blood and muscle during the post-moult period, expressed as grams P per 100 millilitres of blood or 100 grams of muscle tissue.

Explanatory	Sex	Serum	Whole blood		Muscle	
		Total P	Inorganic P	Total P	Inorganic P	Total P
"Paper shell"	Male	0.0023	0.0007	0.0047	0.17	0.27
"Paper shell"	Female	0.0026	0.0009	0.0034	0.20	0.30
"Paper shell"	Female	0.0029	0.0009	0.0039	0.15	0.25
"Rubber shell"	Female	0.0047	0.0019	0.0065	0.17	0.29
"Rubber shell"	Male	0.0042	0.0011	0.0060	0.14	0.25
"Rubber shell"	Male	0.0043		0.0079		
"Rubber shell"	Male	0.0031	0.0016	0.0056	0.16	0.27
"Rubber shell"	Female	0.0049	0.0012	0.0081	0.18	0.26
"Rubber shell"	Female		0.0029	0.0091	0.17	0.30
"Rubber shell"	Female		0.0036	0.0087	0.17	0.30

DISCUSSION

A larger number of observations, particularly during the periods immediately preceding and immediately following the moult, would have been desirable. Difficulty was experienced, however, in obtaining lobsters during those periods, due to the fact that "shedders" and "rubber shelled" lobsters are of little or no commercial value, and are therefore discarded by fishermen and commercial dealers. It is an accepted fact that "shedders" and "soft-shelled" lobsters cannot bear much handling or transportation. This, added to the difficulty of shipment during the summer months when moulting is most prevalent, made it extremely difficult to obtain live "shedders" and soft-shelled lobsters from outside sources. It may be concluded, however, from the analyses that the concentration of inorganic and organic phosphorus in lobster muscle was relatively unaffected by the moult cycle, and that while there appeared to be a slight increase in inorganic phosphorus during the pre-moult period the increase was not greater than prevails within these periods, and was not significant.

Most significant and interesting is the effect of moulting on the concentration of inorganic and total phosphorus in the blood. If the phosphorus content of the blood during the inter-moult period be taken as the normal level, it will be seen that preceding ecdysis there was a sharp rise in blood phosphorus. There is evidence also of a continued rise in blood phosphorus with the approach of ecdysis, probably reaching a maximum at the time of moulting, although it was not found possible to make analyses to support this theory. It was observed, however, that the lobster with the highest concentration of phosphorus in its blood, 0.027 per cent, exhibited to a greater degree than all others examined the characteristics of a lobster soon to moult. Following ecdysis there was a rapid fall in the concentration of phosphorus in the blood, the lowest levels being recorded from "paper-shelled" lobsters. That of "rubber-shelled" lobsters was considerably higher. Thus the analyses indicate that there was an increase in the phosphorus content of blood and blood fractions during the pre-moult period, and that it reached a peak immediately preceding the moult. The values were lowest immediately following the moult, then slowly increased with the hardening of the shell toward what is regarded as the normal level of concentration—that of the inter-moult period. The range in total phosphorus concentration of the whole blood was from 0.0034 per cent, the lowest value obtained from "paper-shelled" lobsters, to 0.0272 per cent, the highest concentration during the pre-moult, with an average concentration during the inter-moult of 0.0128 per cent. It was also noted that the total volume of blood obtainable by breaking a claw was much greater during the pre-moult and post-moult periods than during the inter-moult. It is a conservative estimate that the blood volume was doubled during those periods. Drilhon (1935) noted that there was a marked increase in blood volume in the spider-crab, *Maia squinado*, during the moult period. But in contrast to that of the lobster, Drilhon (1933) observed from analyses of the same species that the total phosphorus in the blood was constant before, during, and after moulting, and that it was almost twice as high in females as in males. Moreover, she found that while there was an increase in inorganic phosphorus before moulting, it was at the expense of organic phosphorus. No correlative changes were evident in lobster blood.

It is commonly observed that during the pre-moult period there are present in lobsters two white masses known as gastroliths, one on either side of the stomach. As already observed, at this time the old shell becomes brittle, due to the absorption of organic and inorganic matter from it. It is a matter of controversy whether the gastroliths, chiefly composed of calcium carbonate and calcium phosphate, "represent a store of lime and other minerals reserved from the old shell for the immediate hardening of the new," or whether they represent the lime removed from the old shell previous to the moult, and are organs for the excretion of lime, playing no role in the formation of the new shell (Herrick 1911). The theory is advanced here that the high concentration of phosphorus in the blood during the pre-moult is caused by the removal of calcium salts from the shell, to be excreted, or deposited in the gastroliths. Soon after the lobster moults the gastroliths are released from the stomach walls, disintegrate, and the carbonate and phosphate of which they are chiefly composed are probably

used for the construction of the new shell. It would seem also that the blood furnishes at least a part of the phosphate necessary for the formation of the new shell, and that immediately following ecdysis both organic and inorganic phosphorus are withdrawn from the blood. The fact that both the gastroliths and the blood can supply but a small fraction of the salts necessary for the building of the new shell in no way invalidates the theory that a part is supplied by them. Thus the blood, as well as the gastroliths, may act as a source of phosphorus at the time when it is most urgently needed for construction of the new shell. This would account for the low concentration of phosphorus in the blood soon after moulting takes place.

There is no evidence from tables I, II and III that inorganic phosphate from the muscle makes any definite contribution to the formation of the new shell. The possibility of error due to the breakdown of labile, organic phosphorus compounds of the muscle, arginine phosphoric acid, hexose-phosphate and adenyl pyrophosphoric acid, to inorganic phosphorus during the analyses was reduced to a minimum by rapid handling of live muscle. One might reasonably assume, therefore, that the error should always be of the same order, and that any appreciable deposition of the inorganic phosphate in the muscle during the pre-moult, or withdrawal during the post-moult, would be reflected in the analyses. Moreover the concentration of both inorganic and total phosphorus in the muscle is relatively constant throughout the whole cycle. One can conclude, therefore, that the flexor muscle, though having twenty times as great a concentration of inorganic phosphorus as the blood, apparently is not utilized as a source of phosphorus during the post-moult period.

SUMMARY

No significant variation in the inorganic and total phosphorus content of lobster muscle during the moult cycle was observed, but there was a relatively wide variation in the inorganic and total phosphorus content of the blood and blood fractions. The hypothesis is advanced that the high phosphorus content of the blood during the pre-moult is due to the removal of phosphates from the shell preparatory to moulting. These compounds are excreted or stored in the gastroliths, and apparently small amounts are stored in the blood. It is suggested that the low concentration of phosphorus in the blood following ecdysis is due to the removal of phosphorus for the construction of the new shell.

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Diurnal Fluctuations in the Upstream Migration of Coho and Spring Salmon

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ABSTRACT

At Skutz falls, Cowichan river, B.C., fish migrated mainly during daylight hours. No correlation was found between diurnal fluctuations in number of migrants and volume or temperature of the water. Light seemed the determining factor. A moderate amount of artificial light had no effect on night migratory movements.

During the autumn of 1939 the size and character of the salmon run of the Cowichan river, Vancouver island, were investigated. General observations were made at the river mouth and at several localities between this point and Cowichan lake, approximately forty-five kilometres upstream. The run was examined in greater detail at Skutz falls, about thirty-two kilometres from the river mouth. Repeated counts of the salmon passing the falls soon revealed that marked diurnal fluctuations occurred in the number of fish moving upstream.

GENERAL CHARACTER OF THE MIGRATION

Throughout August and early September the Cowichan river remained at its low summer level. During this period a few spring salmon (*Oncorhynchus tschawytscha* (Walbaum)), mostly two year old "jacks", appeared in the river. On September 15, following a slight rise in water level, spring salmon, large and small, left the tidal pools in some numbers and went up the river. The first heavy upstream runs of this species, however, and of cohos (*O. kisutch* (Walbaum)) took place from October 18 to 21, coinciding with a large increase in river volume. The advance elements of this influx reached Skutz falls on October 20 and 21. Fish were numerous up to Cowichan lake by October 23. The cohos were definitely not ripe since most of the spawning took place between November 15 and the end of December.

It may be added that observations made in subsequent years have indicated that the migration of 1939 followed a typical course.

OBSERVATIONS AT SKUTZ FALLS

The period of inspection at Skutz falls extended from October 19 to November 10, by which date the greater part of the run had passed this point. Most of the salmon by-passed the falls by travelling up a fish ladder or an adjacent small channel, in both of which counting was relatively easy.

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About seventy-nine per cent of the run consisted of cohos, the rest being spring salmon. As already indicated, the majority of the fish were by no means fully ripe when they passed the falls.

The accompanying graph (figure 1) shows the number of fish per hour passing the counting points during the first nine days of the main migration and during a later period of two days. In the intervening period, omitted from the graph, fish only ran in very small numbers and counts were not made in a comparable manner. During November 7, 8 and 9, counts made by daylight showed a trend quite similar to that prevailing on previous days but since night counting was largely discontinued these records are also omitted from the figure. The usual period of counting was either thirty minutes or one hour.

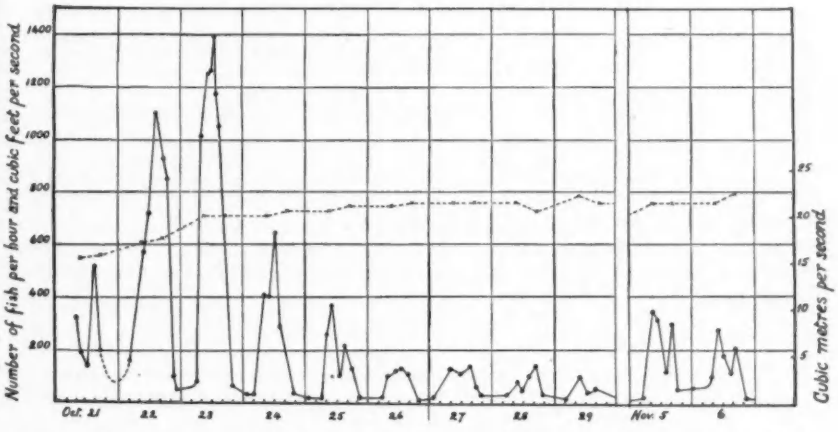

FIGURE 1. Diurnal record of salmon migration (solid line) and river discharge (broken line). The time scale represents four-hour intervals.

It will be seen that the maximal numbers were recorded during the daytime. In several instances the daily record shows a single peak, occurring between 12.00 noon and 3.00 p.m., this being particularly noticeable on the two days which provided the largest number of fish. On other occasions two peaks were obtained, one usually occurring between 7.00 a.m. and 10.00 a.m., the other between 3.00 p.m. and 5.00 p.m. In all cases the number of migrating fish fell to a low level during the hours of darkness, which extended roughly from 6.00 p.m. to 6.00 a.m.

The curve in figure 1 represents the numbers of fish of both species. During the greater part of the period of observations cohos and spring salmon could be readily distinguished, and were recorded separately, except in counts made at night. For the whole period it was estimated that about seventy-nine per cent of the run consisted of cohos, twenty-one per cent being springs. Daily estimates are presented in the following table.

TABLE I. Estimated percentages of spring and coho salmon passing counting points at Skutz falls.

Date		*Percentage*		*Weather*
		Spring	*Coho*	
October	21	60	40	Dull, rain.
	22	14	86	Cloudy, light showers.
	23	23	77	Showery, bright intervals.
	24	25	75	Clear in early morning, clouding later.
	25	33	67	Bright morning, becoming cloudy with rain.
	26	28	72	Dull, showery.
	27	17	83	Dull, showery, clearing at night.
	28	49	51	Fair.
	29	44	56	Fine.
November	5	6	94	Rain in early morning, then brighter.
	6	10	90	Rain all day.

DISCUSSION

DIURNAL PERIODS OF ACTIVITY

Ward (1930) reports that sockeye

"are most active in the morning and evening hours, when the light is moderated, and also on days that are cloudy or dark. One period of greatest activity follows the early morning and along towards 10 A.M. all activity dies down, so that, as a rule, the salmon may be seen, if at all, during midday hours only resting in deep shady spots in the current of the stream. They resume their activity again later in the afternoon, when the sun has moved down far enough to eliminate direct and intensive illumination of the water."

In another paragraph he says

"Observations on the Skagit . . . showed that migrating sockeye moved during the night or the morning and evening hours and lay quietly in deep pools during the more sunny periods of the day. Here, again, it is difficult to say whether the activity is influenced by light or heat, or both together."

In a later paper (1938), however, he states that

"the fish move upstream by day and rest by night but are quiet or less active during the midday period when the sun is high."

Counts made at the Bonneville dam on the Columbia river in 1938 and 1939 showed that, on average, only about four per cent of the salmon passing up the ladders did so during the eight hours of night (Holmes and Morton 1939). The species concerned were mainly spring salmon (*O. tschawytscha* (Walbaum)) and sockeye (*O. nerka* (Walbaum)), with small numbers of cohos (*O. kisutch* (Walbaum)) and chums (*O. keta* (Walbaum)).

Chapman (1941) records that at Rock island dam on the Columbia river in 1937 the sockeyes on two ladders "showed a preference for running in the early morning, the number decreasing as the day progressed, but those going through the middle ladder acted in a directly opposite manner, running predominantly in the late afternoon. The chinook and steelhead ran heavily during the middle of the day." He concludes "that light by itself was not of great importance." It is apparent, however, that in this instance, and in some

of Ward's observations, the comparison is between different periods of daylight, not between daylight and darkness, since counts were not made at night. As already mentioned, the Skutz falls fish showed some variation in the time of maximal activity, which, however, was always during the daytime.

The Skutz falls findings on certain days appear to correspond in some degree with Ward's statements regarding a slackening of activity among sockeyes during the middle of the day. The period of relative inactivity, however, was shorter and much less complete than is implied in Ward's remarks, while on other days, as already noted, no such period of reduced activity was observed. No obvious correlation appears to exist between variations in the time of maximal activity and the weather conditions (see table I).

River Discharge

No relation was found between the diurnal fluctuations in the number of migrants and the volume of the river, though the latter, as already indicated, certainly appeared to influence the migration as a whole. Figure 1 shows the discharge of the river during the period in question. This is based on readings taken at the Cowichan Lake Hatchery, thirteen kilometres above Skutz falls. Observations were made at 9.00 a.m. and 5.00 p.m. and can be taken as representing night and day water levels respectively. The volume at Skutz falls is known to follow these readings very closely. In no instance was the water level at 9.00 a.m. lower than that of the previous afternoon and on five occasions was greater. The volume, indeed, tended to rise slightly throughout the period, irrespective of day and night conditions.

Water Temperature

Water temperatures, also taken twice a day, showed an average difference of only 0.2° C. between 9.00 a.m. and 5.00 p.m. It is evident from the graph that fish frequently ran in large numbers at both these times. However, since on various occasions the fish were running heavily well before 9.00 a.m. whereas by 5.00 p.m. or shortly afterward the numbers were definitely falling off, it appears that they began to move actively when the temperature, on average, was slightly lower than at the close of the active period. Moreover, afternoon temperatures during the latter part of the period of observations were considerably (about 2° C.) lower than early morning temperatures near the beginning of the run, yet the fish still ran in good numbers during the daytime. There is nothing in the temperature record to account for the sharp day and night fluctuations in the number of migrating fish.

Tidal Changes

It is possible that tidal changes may influence the time of arrival of fish in the river mouth. If this were the case and they tended to pass upstream immediately at a more or less uniform speed, fluctuations initiated in this manner might persist during the migration up the river. Observations indicated, however,

that the fish left the tidal pools throughout a long period of day and that they travelled at different rates, so that they soon became well distributed over long stretches of the river. Certain individual fish were observed to linger for days below the falls before passing up the fishway. Moreover, observations at the falls showed that many fish were present during the night periods, though few of them were migrating.

Use of Artificial Light

Night counting was performed with the aid of gasoline lamps and flashlights, these being shielded in such a way that the beam was directed along the top of a step over which the fish had to pass, no undue amount of light being allowed to fall on the pool below. Artificial light used in this manner did not appear to have any effect on the number of fish migrating. This belief is based partly on the fact that the rate of arrival showed no evident difference after the light had been in operation for a time, as compared with the rate at the beginning of a counting period. Further evidence was adduced from the behaviour of fish which were sought out and exposed to artificial light. During darkness most of the fish rested or swam slowly about in the quieter pools. A flashlight trained on such fish had no effect. A gas lamp placed at the edge of the water, within one metre of the salmon, sometimes appeared to have a slight repellent effect, some fish tending to move slowly away. The reaction, however, was not well marked. No effect could be detected on fish which were actively migrating. These observations appear to be in accordance with the statement of Ward (1938) that "some experiments made on fish ladders and parts of a stream illuminated by electric lights seem to show that such illumination does not stimulate the fish to continue migrating at night."

Comparison of Species

No marked differences in behaviour between the two species, as regards time of greatest activity, were observed, though the proportions of the two species recorded at individual counting periods varied greatly. On the two occasions (October 26 and 28) when separation of the species was possible as late as 7.30 p.m. it was noticeable that the proportion of springs was greater than that prevailing earlier in the day. The actual numbers of both species, however, had greatly decreased by this time. The general similarity in behaviour can be illustrated by reference to the curves for October 25, November 5 and November 6. These curves are all very similar in form and height, although the proportion of springs was estimated to be thirty-three per cent on the first date and only six per cent and ten per cent on the other two days.

CONCLUSIONS

Migrating coho and spring salmon at Skutz falls, Cowichan river, moved upstream mainly in the daytime.

Within the daylight period, either one or two peaks of major activity were found.

No marked differences were observed between coho and spring in respect to diurnal periods of activity.

No correlation was found between diurnal periods of migratory activity and river discharge, water temperature or tidal changes.

Artificial light, as used, did not appear to influence migratory movements at night.

Light conditions during the twenty-four-hour day appeared to have a very considerable effect on the movements of the fish.

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Tagging Experiments on British Columbia Pilchards

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ABSTRACT

Internal iron tags recovered by magnets demonstrate general movements of pilchards (*Sardinops caerulea* (Girard)) from Canadian to California fishing grounds. Consideration of recoveries in respect to tonnages of fish landed, size of fish landed, and efficiency of recovery show that in the first year after tagging, tagged fish are more concentrated on northern fishing grounds than on those of California. After the first year the reverse is true. Canadian tags during their first year are more concentrated on the fishing grounds off San Pedro than off central California. Later this condition is not observed. Fish of the 1939 year-class present in California waters in 1940 were taken by the Canadian fishery in 1941. The dates of recovery of first year tags on the United States coast indicate a rather orderly migration. Minimum estimate of the fastest rate of migration indicated gave 14.3 km. per day. In contrast to the inferred condition on major migrations, pilchard movements on northern fishing grounds are very confused. Definite movements from the Washington coast to the Canadian coast are demonstrated. Fish taken in the inlets of Vancouver island during the winter months are part of the general pilchard population. The total mortality rate of the large pilchards tagged in connection with Canadian fishing operations is about 71% per annum.

The Canadian pilchard fishery is pursued principally during the summer and early autumn, commencing in June or July and continuing until September or October. Occasional catches are made in the winter months during the herring season and in 1941-1942 a considerable fishery was conducted during the winter. However, in general pilchards are not found within reach of the fleet fishing from British Columbia ports from November until May.

Where are the pilchards during the period when they are not detected by Canadian fishermen? Of the various possibilities, the one that the pilchards migrate southward where they were exploited by the California winter fishery for large sardines was supported by several lines of circumstantial evidence (Clark 1935, 1940, Hart 1938a). The hypothesis was accordingly tested by a series of tagging experiments which were begun in 1935 and which were continued until 1941.

METHODS

The experiment employed, with modifications, the methods recommended by Rounsefell and Dahlgren (1933) and Dahlgren (1936). Magnetic belly tags were used and were recovered by the use of electromagnets.

Fish for tagging were obtained from the fishermen on the fishing grounds. After various preliminary experiments it was found that under the limitations

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of the work tagging could be carried out most effectively direct from the seine between the time that the net was pursed and the time that the fish were dried up in the bunt end of the net. A few fish were cut off in a pocket of the net close to the skiff from which the tagger worked. From this pocket they were removed one at a time (by the use of a small dip net) for tagging.

Nickel-plated iron tags were used. These were 19 mm. long, 4 mm. wide and 0.86 mm. thick, with ends and edges smoothly rounded, and weighed 0.44 grams. Each tag bore identifying marks.

The tags were inserted in the fish through a hole cut into the abdominal cavity. In some cases the hole was made in the fish by the use of a scalpel having its cutting edge at the end and the tag was inserted by hand (Hart and Tester 1937) but usually the tagging was carried out by the use of a "gun" which combined the functions of cutting the fish, inserting the tags and storing one hundred tags in a magazine (Hart 1938b, Hart and Tester 1938). Tests indicated a negligibly greater efficiency for the gun method of tagging (Hart 1939).

Tags were recovered at reduction plants by the use of electromagnets installed in the bottoms of the chutes in which the meal slides into the grinders after having passed through the driers (Hart 1937). In British Columbia, tags were removed from the magnets by plant employees who, for a reward of fifty cents, turned them in with information concerning the fish being processed at the time the recovery was made. In California, all the scrap metal which accumulated on the magnets was saved for examination by an employee of the Division of Fish and Game who removed the tags (Janssen 1937). Other arrangements which bring the tag and the information concerning the circumstances of its recovery to the proper destination are employed in Oregon and Washington. A few tags were recovered from the bases of grinders, crevices in the machinery, etc.

The method is subject to various errors which must be taken into consideration in making calculations and in reaching conclusions:

(1) No doubt considerable numbers of fish die directly or indirectly as a result of tagging manipulation, and difficulties from this source are amplified by the fact that unavoidable differences in tagging conditions probably affect this mortality to a considerable and unknown extent.

(2) All the tags which enter reduction plants in pilchards are not recovered. Some evidently slide over the magnet without being arrested. Others are lost, permanently held up, or destroyed in their passage through the plant, and some which do adhere to the magnets fail to be returned by the plant crews. The efficiency of plants in recovering tags has been tested (Brock 1940, Hart 1938c, Janssen 1938b, Shuman 1939) in such a way that deficiencies in the method may be compensated for quantitatively.

(3) Not infrequently tags are held up in their passage through a reduction plant. Accordingly, they do not reach the recovery magnet until some time after the fish in which they entered the plant have been processed, with the result that the tags are recorded erroneously. In using tag recoveries to trace minor movements such a possibility must be continually kept in mind.

TAGS USED

During six years 20,391 pilchards were tagged in connection with the regular fishery based on the west coast of Vancouver island as follows: 1935, 978; 1936, 2,535; 1937, 6,936; 1938, 4,982; 1939, 2,370; 1940, 2,590. Of these, 14,185 tags were used in connection with Canadian fishing operations carried on off the coast of Washington and Oregon, as follows: 1937, 6,736; 1938, 2,489; 1939, 2,370; 1940, 2,590. The remainder of the tags were used either off the Vancouver island coast or in the inlets of the west coast of Vancouver island. Further details of the tagging may be found in Hart (1937, 1938b, 1939, 1940, 1941, 1942).

TREATMENT OF DATA

To make certain comparisons between tag recovery results for different fishing areas and different seasons, allowances must be made for several sources of inconsistency in estimates of the concentration of tags among the fish.

Due allowance must be made for the size of the catch, since it is evident that the more fish that are caught the greater the opportunity to recover tags. Accordingly, the numbers of short tons of fish caught have been tabulated for each fishing area and season. To provide an alternative basis for calculation, the number of pilchards caught at each port has been calculated on the basis of the number of fish per ton as calculated from sampling.

Compensation must be made also for the size distribution of the catches in different fishing areas. During Canadian tagging operations pilchards less than 220 mm. standard length were not usually encountered (Hart 1938a) and so were not liable to be tagged. In actual practice, such small fish as were found were discarded. For these reasons fish less than 220 mm. were excluded from consideration. This was done by making use of length distributions obtained by sampling the commercial catch at the various ports. The tons and numbers of fish more than 220 mm. at each port were calculated and tabulated.

Further adjustments were made for the efficiency of recovery. On the basis of tests made on the various plants with known numbers of tags (Brock 1940, Hart 1938c, Janssen 1938b, Shuman 1939) the mean efficiency in each fishing area was estimated. Using these estimates calculations were made of the equivalent tonnages and numbers of fish more than 220 mm. long which would have yielded the same numbers of tags if recovery had been perfect in each fishing area. That is to say, the tonnages and numbers more than 220 mm. long were multiplied by an efficiency factor based on unity as perfection.

Final results were calculated in two forms. The weighted concentrations of tags per 100,000 metric tons were calculated by dividing 100,000 times the numbers of tags recovered in each fishing area by 0.907 times the numbers of short tons of fish caught adjusted for size distributions and recovery efficiencies. The weighted concentrations of tags per 100,000,000 fish were calculated by dividing 100,000,000 times the numbers of tags recovered in each fishing area by the numbers of fish caught adjusted for size distributions and recovery efficiencies.

In combining results for years and places care was taken to avoid averaging averages or concentrations directly.

TABLE I. Data bearing on the recovery of tags out more than three months.

Fishing region and season	Short tons pilchards regarded as caught	Estimated no. pilchards caught	Estimated no. pilchards more than 220 mm. standard length	Estimated no. short tons pilchards more than 220 mm. standard length	No. pertinent tags recovered —— No. years tags out indicated in brackets	Estimated recovery efficiency
BRITISH COLUMBIA						
1937	48,080	205,000,000	203,000,000	48,000	2 (1)	72
1938	51,770	202,000,000	202,000,000	51,000	10 (1)	65
1939	5,523	22,000,000	22,000,000	5,000	2 (1)	70
1940	28,770	139,000,000	128,000,000	28,000	2 (1)	73
1941	51,553	281,000,000	181,000,000	38,000	2 (1)	40
WASHINGTON						
1937	17,200	73,000,000	72,000,000	17,000	0	43
1938	26,480	109,000,000	108,000,000	26,000	1 (1)	43
1939	17,760	74,000,000	71,000,000	17,000	4 (1); 2 (2)	72
1940	798	4,000,000	4,000,000	750	0	50
OREGON						
1937	16,660	73,000,000	71,000,000	16,000	0	18
1938	16,990	71,000,000	70,000,000	17,000	1 (1); 1 (2)	55
1939	22,330	108,000,000	95,000,000	20,000	0	30
1940	3,200	16,000,000	15,000,000	3,000	0	30
WASHINGTON AND OREGON						
1941	33,659	299,000,000	49,000,000	9,000	0	40
CALIFORNIA						
San Francisco						
1936-37	375,037	2,227,000,000	1,337,000,000	257,000	2 (1)	31
1937-38	199,831	1,200,000,000	622,000,000	120,000	0	42
1938-39	200,412	1,422,000,000	462,000,000	94,000	8 (1); 2 (2)	52
1939-40	211,472	1,549,000,000	214,000,000	40,000	2 (2); 1 (4)	44
1940-41	117,869	888,000,000	114,000,000	20,000	1 (3)	57
Monterey						
1936-37	206,226	1,270,000,000	612,000,000	116,000	0	39
1937-38	103,991	721,000,000	210,000,000	40,000	1 (1); 1 (2)	50
1938-39	180,009	1,518,000,000	220,000,000	44,000	5 (1)	64
1939-40	227,217	1,721,000,000	195,000,000	36,000	1 (1); 1 (3)	58
1940-41	165,164	1,420,000,000	154,000,000	25,000	0	65
San Pedro						
1936-37	137,917	879,000,000	430,000,000	81,000	3 (1)	34
1937-38	109,828	875,000,000	214,000,000	40,000	3 (1)	45
1938-39	146,037	1,396,000,000	191,000,000	38,000	6 (1)	47
1939-40	93,083	861,000,000	57,000,000	11,000	1 (2)	36
1940-41	174,781	1,728,000,000	54,000,000	9,000	0	57

In some cases incomplete data have made it necessary to use estimates in the calculations. The situation in all of these cases was such that final results would be only slightly affected by considerable errors in the estimated values.

Some discussions in the paper involve consideration of the number of tags recovered. The weighted numbers in such cases have been obtained by multi-

plying the actual number of recoveries by the reciprocal of the recovery efficiency for the appropriate year and fishing area.

RESULTS

Movements between Canadian and Californian Grounds

The work carried out has resulted in the recovery of 481 Canadian tags at plants situated in the following political divisions: British Columbia, 357; Washington, 53; Oregon, 33; California, 38. (Hart 1937, 1938b, 1939, 1940, 1941, 1942.) A map of the coast forms figure 1.

A very considerable number of the British Columbia, Washington, and Oregon recoveries were made very soon after tagging was carried out. A few of the quick British Columbia returns represent fish which were observed to re-enter the pursed net by jumping over the cork line after tagging and release. Most of the quick returns, however, appear to be due to restoring tagged fish to shoals which were temporarily more available to the fishery than the general population. Data based on these recoveries are not accordingly representative of conditions among pilchards generally. Since it is impossible to determine the proportion of tags which would be recovered if mixing of tagged individuals with the general population were random, all tags recovered in the season and region of tagging are regarded as quick recoveries not to be used in making calculations. The numbers of such quick recoveries were: 1936, 18; 1937, 59; 1938, 268; 1939, 28; 1940, 41.

In addition to Canadian tags, magnets installed in British Columbia plants recovered 127 (+7) tags used by the California Bureau of Marine Fisheries off the California coast (Janssen 1938a, 1938b, 1939, etc.) and 7 (+4) tags used by the Oregon Fish Commission in connection with operations of the Oregon fishing fleet (Brock 1940, etc.).

During and since the preparation of this paper a number of additional tags have been recovered, which have been entered in the appropriate places with a plus sign in brackets. They are used in the calculations when numbers of recoveries are dealt with but are not considered in calculating weighted concentrations where their inclusion would lead to distorting the results, or in other calculations.

The recovery of 38 Canadian tags by plants situated in California and of 127 California tags by plants situated in British Columbia shows beyond doubt that Canadian pilchards move to California and that fish tagged in California waters migrate as far as the Canadian coast. It is evident that the movements indicated are not the exceptional migrations of atypic individuals, since the number of California recoveries of Canadian tags (38) is comparable, not with the number of British Columbia recoveries (357) which includes 337 quick returns, but with the total number of northern recoveries made in the seasons subsequent to tagging. This number is 29: 20 recoveries were made by British Columbia plants (including 2 recoveries made in 1936), 7 by Washington plants, and 2 by Oregon plants, showing more northern tags recovered by the California fishery than by the summer fishery to the north.

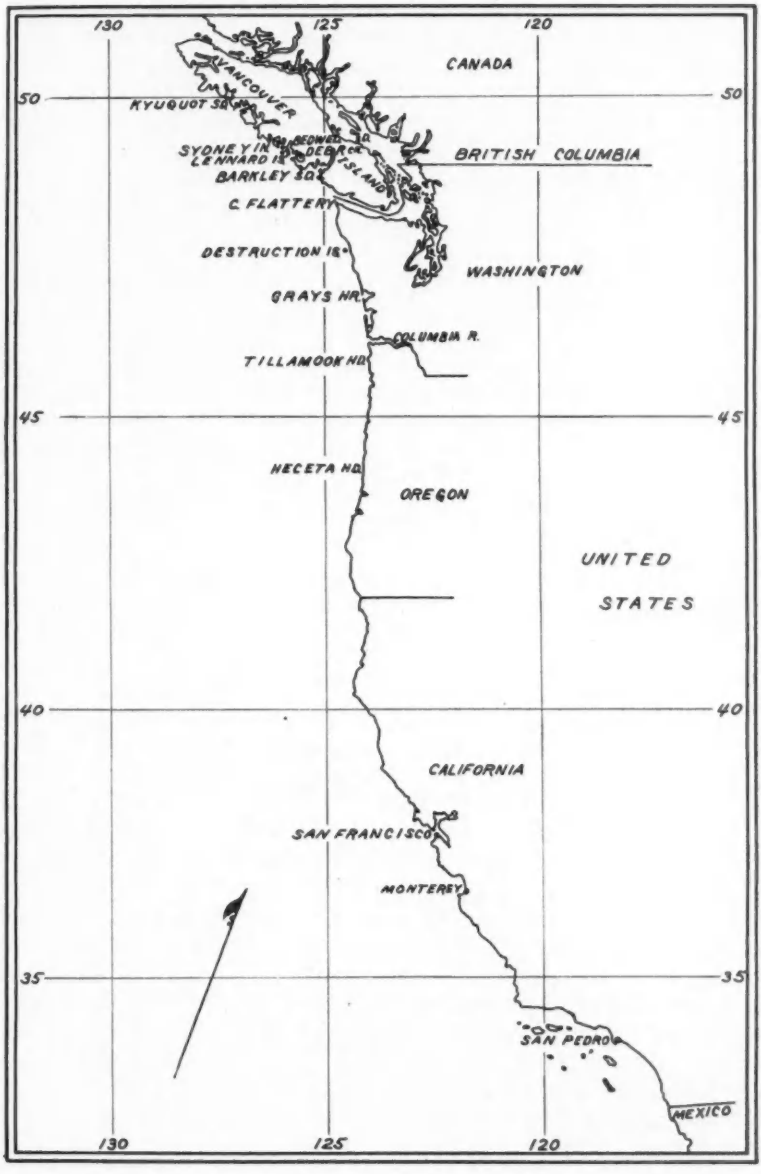

FIGURE 1. Map of Pacific coast of North America, indicating positions of places referred to in text.

COMPARATIVE CONCENTRATIONS ON WHOLE COAST

The numbers of returns indicated above are too small to permit confident analyses. However, certain indications are sufficiently interesting to warrant discussion.

The weighted concentrations (page 166) for British Columbia, Washington and Oregon grouped together under the heading Northeast Pacific, and for all California ports for the years 1936-1937 to 1940-1941 combined were:—

	Tags recovered	Tags per 100,000 metric tons, weighted	Tags per 100,000,000 fish, weighted
Northeast Pacific.....	27(+1)	18.4	3.9
California.....	38(+2)	10.3	1.8

These figures include recoveries made in the first season following that of tagging and in subsequent seasons. When these are separated the following results are obtained for first season after tagging only:—

	Tags recovered	Tags per 100,000 metric tons, weighted	Tags per 100,000,000 fish, weighted
Northeast Pacific.....	24	16.4	3.5
California.....	29	7.8	1.3

These two sets of data show a considerably higher concentration of tags in the northeast Pacific area than in California waters. The following possible explanations of the difference suggest themselves: (1) The excess of tags from the northeast Pacific may be due to tags which have been held over in the reduction plants from the numerous quick recoveries immediately following tagging in the previous year; (2) the difference may depend upon errors in weighting and particularly upon those resulting from different length distributions of fish on different fishing grounds; (3) the differences may be due to the fact that migration from northeast Pacific to California waters is incomplete.

In many cases it is difficult or impossible to be certain from the appearance of a tag and the circumstances of its recovery whether it has been held up in the reduction plant in one season to be recovered in the next. As tags are known to be returned a year or more after they have entered the plant it is accordingly likely that some of the tags reported as having been out for a year have actually been quick recoveries which took many months to pass through the reduction machinery. On the other hand it is not believed that the greater concentrations of older tags in the northeast Pacific fisheries is entirely or largely due to technical shortcomings. Usually pilchards have been tagged during the middle or early part of the run so that the chances of recovery during the season would be great. Most of the reduction plants which have produced pilchard tag recoveries have operated on herring between pilchard seasons and that operation would reduce the number of belated recoveries. Finally, examination of the data shows that the number of recoveries of tags out more than a year is related to the tonnage of fish caught during the season of recovery rather than

to the number of quick recoveries in the preceding season. It is believed that not more than about four of the recoveries under discussion are the results of hold-overs.

There can be little doubt but that the differences in the weighted concentrations of tags in Canada and California are partly a result of incomplete compensation in the weighting method and the following discussion deals with adjustments which are applicable under two major assumptions. Pilchards tagged in Canada were taken more or less at random from a population with a generally normal distribution around a centre of abundance between 240 and 250 mm. in standard length. British Columbia and the other northeast Pacific recoveries were made from the same or similar populations. California recoveries on the other hand were made from a population with an obviously greater length spread about a lower centre of distribution. (See figure 2.)

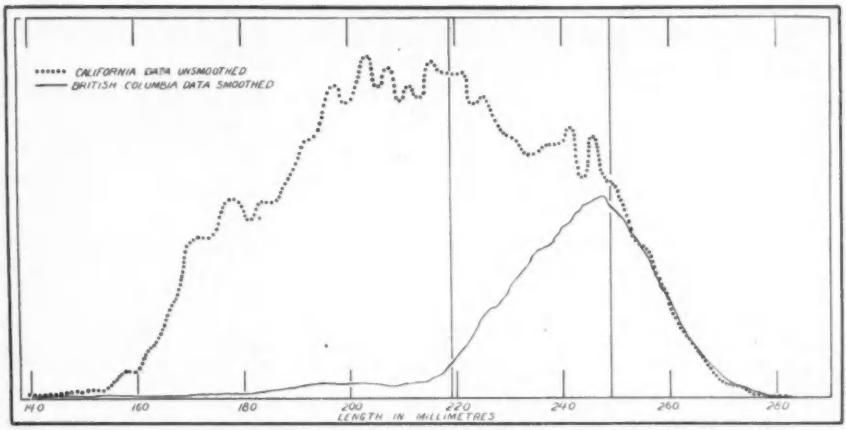

FIGURE 2. Comparison of length frequencies of pilchards in British Columbia and California. British Columbia data: complete seasons 1937-1941; each season given equal weight. California data: winter fisheries in lunar month most closely corresponding to mid-December to mid-January for San Francisco and Monterey, and for lunar month most closely corresponding to February and March for San Pedro; seasons 1936-37 to 1941-42; seasons at each port weighted according to intensity of sampling; ports weighted approximately according to landings, approximately 4 : 3 : 2, respectively; data recorded in 2 mm. intervals, not otherwise smoothed.

In figure 2 representative length frequencies for California and Canadian pilchard catches have been weighted so that the numbers of fish more than 249 mm. are the same in each case. This treatment may be justified on the assumption that (1) there is no length differentiation against fish more than 249 mm. taking part in the summer northward migration such as is evident for the lesser lengths. From figure 2 it is evident that (2) if catches represent population even approximately, and (3) if the populations of fish more than 249 mm. standard length correspond in abundance (actually, this condition is not met, but the weighting resorted to in calculating weighted concentrations

produces for present purposes the effect of equality) there must be a considerable dilution of Canadian tags among fish more than 219 mm. in standard length as the schools of fish from Canadian waters enter California waters. Calculation based on the data from which the figure was prepared shows that under these conditions—number of fish more than 219 mm. standard length in California: number of fish more than 219 mm. standard length in Canada : number of fish more than 249 mm. in each locality :: 1.68 : 1 : 0.38. If the weighted concentrations of tags for California are readjusted by multiplying the values 10.3, 1.7, 7.8, and 1.3 shown at the first of the subsection by 1.68 they are brought closer into line with those for the northeast Pacific giving 17.2, 2.9, 13.1, and 2.2 as compared with 18.4, 3.9, 16.4, and 3.5. This method of re-weighting the material involves the tacit assumption that the particular fish which were in Canada in one season return there during the following year—an assumption which could be made only with reluctance.

If an alternative assumption is made, that a random selection of the pilchards between 219 mm. and 249 mm. move to Canadian fishing grounds each year, it may be shown from figure 2 that the concentration of tags in Canada would be only 1.13 that in California waters and that the number of California pilchards in the length range as weighted would have to be between five and six times the Canadian number in order to completely explain the differences in weighted concentrations.

These estimates are based on the following calculation using the length distributions shown in figure 2.

In addition to the assumptions above assume: (4) Canadian tags are distributed randomly throughout the Canadian population more than 219 mm. long; (5) initial mortality due to tagging has come to an end at the time southward migration begins; (6) one unit of tags is in the Canadian population at the commencement of the southward movement—and the concentration is designated as 1.00.

The values graphed in figure 2 indicate that: above 249 mm. standard length 0.38 units of tags will occur; and below 249 mm. standard length 0.62, (1.00-0.38) units of tags will occur.

If all the tagged fish enter the California fishing grounds the concentration there will be $1/1.68 = 0.60$, but the concentration of tags between lengths 219 mm. and 249 mm. will be $0.62/1.30 = 0.48$.

If all the tagged fish more than 249 mm. standard length return to Canada along with a random selection of the fish less than 249 mm. standard length, the number of units returning to Canadian fishing grounds will be

$$0.38 + 0.62 \times \frac{0.62}{1.30\ (=1.68 - 0.38)} = 0.68$$

and the ratio concentration of tags in Canada : concentration of tags in California is 1.13 : 1.00 (=0.68 : 0.60).

Values of the ratio, fish more than 219 mm. standard length in California : fish more than 219 mm. standard length in Canada corresponding to various ratios, weighted tag concentrations in Canadian catches : weighted tag concentrations in California catches can be determined graphically by plotting values of the latter ratio calculated from the formula

$$p\left(0.38 + \frac{0.384}{p - 0.38}\right) \text{ when } p \text{ is former ratio.}$$

It is possible that the true situation lies between the two assumptions outlined above and that the proper proportion for adjustment of the results lies between 1.68 and 1.12.

From figure 2 can be seen the reason for the difference in the relationships between the two weighted concentrations, i.e. the average weight of California fish more than 220 mm. standard length is less than the average weight of Canadian fish more than 220 mm. standard length.

Incomplete migration from Canadian fishing grounds to those of California explains part of the difference observed as will be shown later in the subsection dealing with the status of fish taken in the inlets during the winter months. However, it is believed that fish wintering in the inlets cannot explain all or most of the observed differences. To what, if any, extent pilchards remain off-shore in northern waters during the winter cannot be stated at the present time.

In summary: differences between weighted concentrations of tags in northeast Pacific and California pilchard catches are shown to depend upon the existence of hold-overs, the details of the weighting methods used, the influence of fish remaining in the inlets, and may be increased by various errors in assigning weights, etc. All of these sources of difference taken together do not appear to be great enough to produce differences of the extent observed. It would appear, accordingly, that there is some lack of completeness in the return of pilchards to California waters during the first year after their appearance on the fishing grounds of the northeast Pacific.

One further point may be raised in comparing returns from the northern fishing grounds with those from California. Such difference as there is between the regions in the weighted concentrations of tags out for more than a year indicates a greater concentration in the California fishery, viz.:—

	Tags recovered	Tags per 100,000 metric tons, weighted	Tags per 100,000,000 fish, weighted
Northeast Pacific.....	3(+1)	2.1	0.43
California.....	9(+2)	2.4	0.42

The observed difference could have arisen by chance in one out of about five trials and cannot, accordingly, be regarded as significant.

CONCENTRATIONS IN CALIFORNIA

The weighted concentrations of Canadian tags show differences at different California ports. The results for San Pedro are higher than at San Francisco and Monterey. When the weighted concentrations for each year at San Francisco and Monterey combined are compared by pairing with respective yearly concentrations at San Pedro (Fisher 1932, sec. 24) the difference is shown to lie on the 0.05 level of significance. This is the resultant of two opposite tendencies. Tags recovered during the winter following tagging are more abundant in southern California than in central California, viz.:—

	Tags recovered	Tags per 100,000 metric tons, weighted	Tags per 100,000,000 fish, weighted
San Francisco and Monterey areas.....	17	5.5	0.96
San Pedro area.....	12	18.4	3.13

That the difference between the concentrations is significant is suggested by considering the fiducial limits of the number of tags recovered on the reasonable assumption that the numbers of tags recovered in various trials will be a Poisson distribution: for the proportions to be equal 57 tags would have to be recovered from central California or 3.6 from southern California provided that only one number were to be altered. The chances are less than one in one hundred that the former number exceeds 30.7 or that the second be less than 4.9 (Ricker 1937). The consistency of the difference may be tested by the pairing method comparing the weighted concentrations in each season and testing the significance of the annual differences from zero. This indicates a difference which could occur by chance once in about eight sets of trials. It will be observed that the weighted concentration of tags in the San Pedro area is very similar to that observed in the Pacific Northeast region.

Tags recovered more than a year after tagging appear to give no indication of a higher concentration off southern California than off central California, viz.:—

	Tags recovered	Tags per 100,000 metric tons, weighted	Tags per 100,000,000 fish, weighted
San Francisco and Monterey.....	8(+2)	2.6	0.45
San Pedro.....	1	1.5	0.26

The difference between first year returns and those for subsequent years could have arisen by chance since comparing both kinds of returns in the two areas in one operation by the chi-square test indicates that the observed difference in returns between first year and other years could have arisen by chance almost once in twenty trials.

YOUNG OF 1939 YEAR-CLASS

In May of 1940 large schools of small pilchards appeared in Canadian waters in unprecedented abundance. These fish were shown to be of the 1939 year-class (Walford and Mosher 1941). In the autumn these fish largely disappeared from the inlets of the west coast of Vancouver island. In the early summer of 1941 the fish taken in connection with the usual pilchard fishery included a substantial proportion of small fish with standard lengths grouped about 200 mm. These fish also proved to be of the 1939 year-class. The problem arises: were these fish part of some special population confined to northern waters or were they part of the general population? There is evidence for a belief that there was at least some admixture of the general population in the recovery of two tags put out during the preceding year at Monterey by the California Bureau

of Marine Fisheries. One of these was used on May 2, 1940, on a fish of approximately 135 mm. standard length which was, accordingly, almost certainly of the 1939 year-class. The other was used on October 4, 1940, on a fish about 165 mm. in standard length which was probably of the 1939 year-class. The two recoveries indicate a movement of some California fish of the 1939 year-class to the Canadian coast during the early part of 1941. No evidence is available as to whether a part of the fish taken in Canada represent a special northern population.

SPEED OF SOUTHWARD MIGRATION

Canadian tags recovered in California within a year of having been put out have as average dates of recovery: at San Francisco, January 9; at Monterey, January 16; at San Pedro, March 12. The ranges of dates for recoveries are: for ten recovered at San Francisco, December 27 to January 29; for seven recovered at Monterey, December 30 to January 31; for twelve recovered at San Pedro, February 23 to March 25. It is evident that the times of recovery at San Francisco and Monterey do not differ significantly from each other but that both differ significantly from those at San Pedro. The observation is in line with the hypothesis of an orderly migration down the coast.

Canadian tags recovered in California in the second, third, or fourth season after tagging came back on the following dates: San Francisco, four recoveries between December 5 and February 5, average January 17; Monterey, two recoveries December 15 and January 11; San Pedro, one recovery on March 2. Examination of the recovery dates at San Francisco shows that the variation is considerably greater for the tags which have been out longer. Considering $\sigma_\sigma = \frac{\sigma}{\sqrt{2N}}$ (Pearl 1923) the difference is shown to be of a magnitude which would occur by chance once in about seven trials and so cannot be considered as of statistical significance.

Pilchards tagged for more than one season have been shown to behave differently from those out in their first year after tagging in three respects: in their relative abundance in northeast Pacific fishing grounds and those of California; in their relative abundance in central California and southern California fishing grounds; and in the variation in their time of recovery at San Francisco. None of the differences in behaviour are of definite statistical significance taken by themselves. By making use of the relation $\log_e P = -1/2$ chi square for 2 degrees of freedom, they may be combined (Fisher 1932 sec. 21.1) to show that three such differences could arise by chance once in about 24 trials. The existence of a difference at that level of probability indicates the probable existence of an actual difference in behaviour.

Given tagging dates and locations and the approximate date and localities of recovery, minimum speeds of migration can be calculated and the following approximate results in kilometres per day are obtained.

Tagging time and place	Place of recovery		
	San Francisco	Monterey	San Pedro
August 1936 off Vancouver island	7.1, 8.5	—	9.2, 8.7, 8.9
July-August 1937 Washington coast	—	6.3	6.6, 6.8, 6.8
July-August 1938 Washington coast	—	6.8, 8.4	—
September 1938 Vancouver island	11.6, 12.9, 10.6 11.4, 12.4, 13.7	10.1, 11.7, 14.3	12.6, 12.7, 12.4 12.4, 12.1, 11.2
August 1939 Washington coast	—	7.1	—

In spite of the errors inseparable from the method the rates indicated are remarkably consistent within seasons and recovery ports. Some of the differences between seasons are no doubt accountable to the fact that the fish were tagged at the height of the season some time before the commencement of their southern migration. The higher rates indicated for migration to San Pedro in 1936 and 1937 would support this suggestion since the influence of the period when no active migration is under way would have less effect in distorting calculated speeds to that port than for others for which the free time was less. Fishing continued into October in 1936 and 1937 so it may be assumed that no southward mass migration began until then. If forty days and sixty days respectively are subtracted from the migration period, the estimated speeds will be increased by about 30 per cent for San Francisco and Monterey and about 20 to 25 per cent for San Pedro recoveries. Application of these justifiable corrections brings the rates of migration as calculated for different years closer together.

The speeds indicated correspond rather closely with those indicated by Janssen (1937, 1938a) (average approximately 6 nautical miles or 11.1 km. per day) for the northern migrations in 1937 and 1938.

The southward migration down the coast would appear to be at a relatively uniform speed since the difference between average dates for pilchard tag recoveries at San Francisco-Monterey, and at San Pedro, January 12 and March 12, 59 days, divided into the approximate distance between the respective fishing grounds, 560 km., indicates a migration speed of about 9.5 km. per day.

Movements on Canadian Fishing Grounds

For reasons outlined in the section on methods the means of tag recovery is not well adapted to the study of short migrations. In some cases, however, evidence of mixing of fish on the fishing grounds appears beyond question. In 1937, for example, tags used near cape Flattery were recovered around Destruction island and Grays harbour. Some tags used at Destruction island were recovered at the same place two months later, one was taken off the mouth of the Columbia after nine days, and others continued to be taken off the area

between Tillamook head and the mouth of the Columbia river. Nine weeks after tagging another tag used at Destruction island was recovered off Heceta head. Tags used off Grays harbour were recovered later off Destruction island. The returns indicate general mixing. As a further example may be cited the recoveries made in 1938 of tags used in the inlets of the west coast of Vancouver island. Tags used in several of the inlets were reported—apparently validly—from a great variety of other inlets, indicating very general movement along the northwestern part of the Vancouver island coast.

The most interesting results from tagging returns were obtained during some seasons in which marked changes took place in the location of the fishing grounds. In such cases some evidence concerning movements is available and is of interest in that it indicates the movement of fish between widely separate parts of the fishing grounds rather than the appearance on the fishing grounds of new shoals which had not been exploited previously.

MOVEMENTS TO CANADIAN WATERS

During the early summer of 1938 the Canadian pilchard fleet operated off the Washington coast and there was no evidence of fish in the inlets of Vancouver island or within a reasonable distance of the shore. In the middle of the season fishing fell off to nothing and later pilchards were found to be very available in the inlets on the northwestern part of the west coast of Vancouver island. One tag used off the Washington coast was recovered from fish taken on the west coast of Vancouver island at a plant which had not operated during the early part of the season and this recovery showed beyond question that fish had moved from the southern to the northern part of the fishing grounds. Recoveries by other plants of tags used off the Washington coast reported, apparently correctly, from west coast of Vancouver island landings, support the certain return and indicate a general movement of fish. It is worthy of remark that the movement which undoubtedly took place was not observed by the fishermen who were plying between west coast reduction plants and fishing grounds off the Washington coast and it is concluded that the movement took place too far off shore to be observed. This condition would be met if the fishermen restricted their courses to the most direct route between Barkley sound reduction plants and Washington coast fishing grounds and the fish followed the most direct route from the latter to Kyuquot sound rather than follow the coast line.

In 1940 fishing again moved during the season from off the Washington coast to off Vancouver island. In the latter year, however, the transition was less clear cut and the results were very much less satisfactory for the British Columbia fishing industry. In this year tags used off the Washington coast were later reported from off Vancouver island near Lennard island and off the Columbia river. Returns referred to localities near Vancouver island appear to be too numerous and to have been made by too many plants to be the result of technical imperfections and it is concluded that the movement is demonstrated of fish from off the southerly part of the Washington coast to the fishing grounds exploited toward the end of the season off Vancouver island.

Estimates of speed of migration for short distances are more subject to error than those for longer migrations but may be presented for purposes of comparison with the speeds indicated for movements between Canadian and California fishing grounds: from the Washington coast to Heceta head, Oregon, 1937, 6.6 km. per day; from Destruction island to the mouth of the Columbia river, 1937, 18 km. per day; from the Washington coast to Vancouver island, 1938, 7.9 km. per day; from the Washington coast to off Vancouver island, 1940, 5.1, 5.1, 5.3, 7.4 km. per day; from the Washington coast to Lennard island, 1940, 4.8, 5.5, 4.8, 8.0 km. per day; and from the Washington coast to the mouth of the Columbia river, 1940, 4.5, 9.7 km. per day.

FISH TAKEN IN INLETS IN WINTER

Although the main body of pilchards leaves northern fishing grounds in the autumn, some fish frequently remain in the inlets throughout the winter, where they are occasionally captured during the winter herring fishing season and where they sometimes provide early in-shore fishing before the main pilchard shoals approach the Vancouver island coast in the summer. Some of the tag recoveries indicate that these pilchards are a part of the general population cut off from participation in the general movement by local conditions rather than a special local population.

In 1936 tags used on fish released off Vancouver island were recovered in inlets indicating an in-shore movement. During this year 100 tags were put out in Bedwell sound on the west coast of Vancouver island. One of these tags was recovered two days later from the same locality and three months later during the herring season a landing of pilchards from Deer creek yielded another tag, indicating that part at least of the tagged fish were remaining in the inlets throughout the winter. Early in the 1937 season a few pilchards were found in Sydney inlet far removed from the main body of pilchards which did not approach the Canadian coast during that season, and some observers were of the opinion that these fish were moving out from the deeper inlets. This opinion was confirmed by the recovery from these fish of a tag used on a fish released in Bedwell sound during the previous summer.

Mortality Rates

If mortality rates are constant from year to year, and, if the tagged fish after recovering from the first shock of manipulation die at the same rate as untagged individuals, the total annual percentage survival rate, i.e. the complement of the mortality rate, from all causes should be the same as the percentage of tags recovered in any one year is of the number of tags recovered in the year immediately preceding. Consequently, logarithms of tags recovered plotted against year of recovery should lie along a straight line whose slope is indicative of mortality rate and whose y-intercept is the logarithm of the number of tags which could have been expected as recoveries in the first year had there been no sharp break in conditions (figure 3). The numbers of recoveries made have been corrected for efficiency of recovery since cumulative changes in recovery efficiency would have vitiated the results. California recoveries made

in the season immediately following tagging and therefore out for a period between four and seven months have been included with tags recovered in the northeast Pacific after having been out for approximately a complete year. From the line fitted by least squares to the data for all recoveries the annual mortality rate can be calculated as approximately 71 per cent and it will be seen that the number of tags expected for the year of tagging is indicated at 282

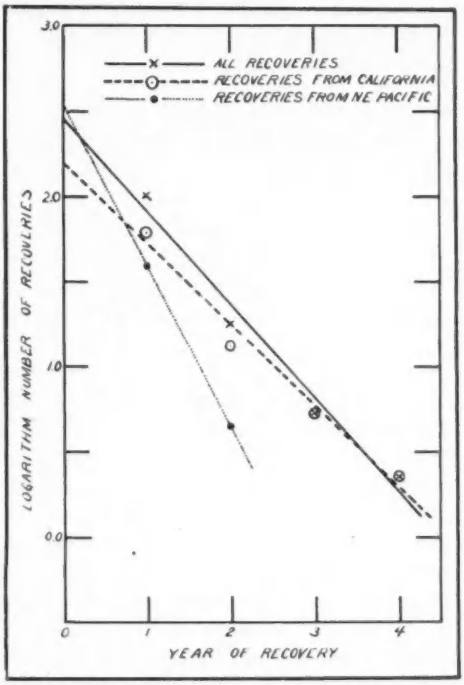

FIGURE 3. Decline in recoveries of pilchard tags.

(= antilog 2.45). This figure may be compared with the number of tagged fish estimated as having been processed, 799, and the difference may be regarded as the number of true "quick recoveries" corrected for recovery efficiency. Considering California returns alone the mortality rate is indicated as being somewhat less that for all the data or 66 per cent per year. The mortality rates so indicated are in reasonable agreement with computations based on age analysis made at the laboratory of the South Pacific Investigations of the Fish and Wildlife Service. The number of recoveries involved is insufficient to assure more than one-figure accuracy in estimated mortalities.

In an earlier report (Hart 1939), estimates of the intensity of the fishing on local parts of the pilchard stock were based on calculations of

$$\frac{\text{number of quick recoveries (corrected for efficiency of recovery)}}{\text{number of tags used}}$$

and it was pointed out that for the season (1938) concerned the resulting 3½ per cent and 17 per cent values obtained for the intensity of fishing on the Washington and Canadian coasts respectively were minimum estimates. The method of Thompson and Herrington (1930) for estimating fishing intensity and residual mortality (= natural mortality?) is not applicable since it depends upon the assumption that early mortality associated with tagging operations is negligible. As it is evident that initial mortality in the pilchard is large, and variable from season to season, no dependable method of calculating the component mortalities accurately is available.

SUMMARY

More than twenty thousand magnetic belly tags were used to investigate movements of pilchards. These tags were recovered by electromagnets situated in reduction plants.

In comparing concentrations of tagged pilchards in different localities consideration was given to the influence on the results of (1) deficiencies in the methods employed, (2) the tonnages of pilchards taken on different fishing grounds, and (3) the length frequency of pilchards taken in different parts of their range.

There is considerable movement of pilchards between northern fishing grounds and those of California. This interchange of individuals is too great to be the result of casual movements and definite migration is indicated.

During the first season following that of tagging, tagged fish appear to be more concentrated on northern fishing grounds than off the California coast. This observation may be accounted for in three different ways and it is believed that each of them plays a part in producing the difference, viz. (1) deficiencies in the method of recovering tags, (2) errors in adjusting for the length frequency of the fish in different localities and (3) migration from northern fishing grounds to California fishing grounds is incomplete during the first year after tagging.

During the first year after tagging, tagged fish are relatively more concentrated on fishing grounds off San Pedro than off Central California. Later this condition does not hold.

Pilchards of the 1939 year-class were unusually abundant in northern waters during 1940. The same year-class was again abundant in British Columbia waters in 1941 and in that year included two fish which were tagged in California during 1940.

During the first year after tagging average dates of recovery of Canadian tags at California ports are as follows: San Francisco, January 9; Monterey, January 16; San Pedro, March 12.

Average minimal speeds of southward migration range from 6.3 to 14.3 km. per day. Errors involved in the method are responsible for part of the variation between the results for different years and to different ports. All errors would tend to make the estimates of speed of migration lower than the correct ones.

Fish move about considerably on northern fishing grounds. In some cases these movements appear to be more or less random, but in others general movements appear to take place which accompany changes in the locality of the

fishing grounds. Part of the general pilchard population moves in and out of the inlets on the west coast of Vancouver island and a few individuals frequently spend the winters in inside waters, thus failing to take part in the general southward migration.

Pilchard tag recovery data can be used in calculating survival and total mortality rates in the species. A total mortality rate of approximately 71 per cent per year is indicated by the data at hand.

ACKNOWLEDGMENTS

The pilchard tagging programme has been financed jointly by the Fisheries Research Board of Canada and the Department of Fisheries of the Province of British Columbia. It has enjoyed the co-operation of the Directors and various members of the staffs of these organizations as well as that of various fishing companies and their employees who have given invaluable assistance in the work of tagging fish and recovering the tags. The state fisheries organizations of California, Oregon, and Washington have shown every courtesy in attending to the recovery of tags and in supplying information concerning the stocks of fish from which the tags were recovered. The California Bureau of Marine Fisheries has shown the further courtesy of allowing first publication of data concerning California tags recovered in Canadian waters in cases where the recoveries appeared to be of particular interest in connection with the supply of fish in northern waters.

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Magnesium-Ammonium-Phosphate Crystal Formation in Canned Lobster

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ABSTRACT

Analyses of canned lobster for magnesium, ammonia, and inorganic and total phosphorus have indicated that the extent of crystallization of magnesium ammonium phosphate in canned lobster is limited by the concentration of magnesium. This has been confirmed by experimental canning. It has been shown by experimental canning that sea-water, the magnesium concentration of which is approximately five times that of lobster meat, is the major contributing factor in the formation of large quantities of these crystals. Abstention from the use of sea-water in any step of the canning process restricts crystal formation to the point where it escapes the observation of the consumer.

Crystals of magnesium ammonium phosphate are sometimes found in various canned marine products. Their presence has been reported in canned shrimp (Purcell and Hickey 1922), in canned salmon (Manley 1931; James 1933), and they have also been found in canned crab and canned "chicken haddies." They have been identified by various investigators, including the author, as hydrated magnesium ammonium phosphate, or "struvite" with the chemical formula $MgNH_4PO_4 . 6H_2O$ (Clark and Clough 1925; Ayres 1942). These crystals are hard and colourless, and, though harmless, are frequently mistaken by the consumer for broken glass. Their presence, therefore, has a definitely adverse effect on the marketing of the canned product.

"Struvite" crystals occur infrequently in recently canned lobster. However, when commercially canned lobster is kept in storage it has been found that a progressive deposition of the crystals occurs, and after a period of eight to ten months, at an average storage temperature of 16° C., they are present in varying amounts in nearly all the cans. Sometimes they are present, particularly when found in recently canned lobster, as minute crystals distributed over most of the surface of the parchment lining of the can; in other cases they are centralized as large orthorhombic and sphenoidal crystals on small areas of the lining and also on the meat.

Several theories have been advanced to account for their formation in canned marine products. Carter (1933) stated that in canned salmon they are "formed during unduly retarded cooling of the cans after processing." Bedford (1935) suggested that their formation is related to physiological peculiarities of the fish, but also that variations in retorting temperature, conditions of cooling,

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and probably careless handling of fresh fish, induce their formation in canned salmon. Wood (1940), commenting on the canning of crustaceans, ascribed the cause of their formation to bacterial action prior to or during storage. Apparently no data have been presented to support these theories. Early in this investigation it became very evident that the conditions outlined above were not the chief factors influencing their formation in canned lobster, though the possibility that they play some small part in their formation has not been excluded. As an approach to the problem, chemical analyses have been made to determine the concentration of constituent ions of magnesium ammonium phosphate—phosphate, magnesium and ammonium—both in the fresh muscle and in the canned product, and experimental canning has been conducted to substantiate and supplement the finding from the chemical analyses.

PHOSPHORUS

The inorganic phosphorus content of fresh lobster muscle ranges from 0.13 to 0.16 per cent and total phosphorus from 0.26 to 0.30 per cent (Hollett 1943). Analyses have been made (results of typical ones given in table I) to

TABLE I. Concentration of phosphorus in canned lobster.

Age of can	History	Pickle		Meat	
		Inorganic P (%)	Total P (%)	Inorganic P (%)	Total P (%)
30 days	Experimental pack, no crystals present		0.13		0.15
40 "	Experimental pack, no crystals present	0.073	0.11	0.070	0.11
45 "	Experimental pack, no crystals present	0.070	0.11		0.13
5 months	Experimental pack, no crystals present	0.10	0.15		0.16
5 "	Commercial pack, no crystals present	0.054	0.08	0.050	
8 "	Commercial pack, few large crystals on lining, few smaller ones in meat	0.053	0.085	0.050	0.15
8 "	Large crystals on two areas of lining, some in meat, commercial pack	0.073	0.11		0.18
8 "	Many crystals over whole surface of lining	0.050	0.08	0.046	
9 "	Commercial pack, many crystals	0.12	0.16	0.11	0.17
9 "	Experimental pack, many crystals	0.11	0.16		0.17
9 "	Experimental pack, few crystals	0.12	0.16		0.175

determine the concentration of phosphorus in the pickle and meat of canned lobster. Most cans were from commercial packs of lobster, canned in various areas and of varying ages. Many factors, such as method of cooking the

lobsters, thoroughness in washing the meat, variation in the amount of pickle added to the can, and presence or absence of the crystals in the can, must necessarily have had a determining influence on the final concentration of phosphorus in the canned lobster. It is not surprising, therefore, that there was considerable variation in the inorganic and total phosphorus content of the pickle and the meat. The analyses indicated that equilibrium existed between the inorganic phosphate content of the pickle and that of the meat. Greatest variation was evident in the inorganic phosphorus of the pickle, the concentration ranging from 0.05 to 0.12 per cent. This variation may be attributed in part to the factors outlined above, but may be due also to breakdown of labile, organic phosphorus compounds of the muscle tissue.

AMMONIA

Method of Estimation

The concentration of ammonia in the pickle of canned lobster was derived by deducting the values for trimethylamine nitrogen from those for total volatile basic nitrogen. Trimethylamine was estimated by the method of Beatty and Gibbons (1936). The method of Conway and Byrne (1933) was used for the determination of total volatile base.

In fresh muscle and in pickle

The data of Reed (1925) show that the concentration of volatile basic nitrogen, which is mainly ammonia nitrogen, in fresh lobster muscle is approximately 0.005 g. per 100 g. In this investigation ammonia was estimated only in the pickle of canned lobster. Because inorganic phosphate was found to be evenly distributed between pickle and meat in canned lobster, and because ammonia diffuses more readily than phosphate, it was assumed that there is equilibrium between the ammonia content of the pickle and that of the meat. Moreover, it was the concentration of the ammonium ions in the pickle rather than in the meat in which we were most interested, since the pickle is the immediate source of the ions which form magnesium-ammonium-phosphate crystals. Analyses were made of the pickle of canned lobster from various canneries throughout the Maritimes (table II). For comparative purposes, ammonia analyses were made of an experimental pack of lobster canned at this laboratory and stored for about six months (table III). Ammonia in the commercial pack ranged from 0.023 to over 0.04 g. nitrogen in 100 ml. of pickle. These values compare favourably with those of the experimental pack in which the ammonia nitrogen content ranged from 0.022 to over 0.04 g. per 100 ml. of pickle. The minimum concentration of ammonia nitrogen in pickle from recently canned lobster was found to be 0.017 per cent. This represents a value over three times that of recently cooked lobster meat.

It may be noted in passing that there is a slight increase with age both in trimethylamine and ammonia in canned lobster.

TABLE II. Magnesium and ammonia content of commercially canned lobster.

Age of can (months)	Grams per 100 ml. of pickle		
	Magnesium	Ammonia nitrogen	Crystals
6	0.014	0.033	None
6-7	0.0275	0.029	Many
6-7	0.022	0.033	Very many
6-7	0.026	0.034	Many
6-7	0.0275	0.023	Many
6-7	0.0217	0.022	Many on lining
8	0.0115	0.031	Many small
8	0.014	0.038	None
8	0.016	0.032	None
8	0.016	0.022	Many tiny
16	0.012	0.032	On lining and meat
16	0.011	0.037	Some
17	0.010	0.04+	Many on lining
17	0.010	0.028	Some large

MAGNESIUM

METHOD OF ESTIMATION

Magnesium was estimated photometrically by the method of Gillam (1941). Digestion of organic matter was effected by heating with concentrated perchloric and nitric acids. The method is rapid and particularly suitable for the estimation of magnesium in biological material, since other ions are present in too low a concentration to cause interference in the pink colour developed by magnesium. Precipitation of calcium, iron and phosphates is therefore unnecessary.

IN LOBSTER MUSCLE

Analyses of the flexor muscle of the American lobster indicated that the concentration of magnesium varied from 0.0227 to 0.0234 g. in 100 g. of muscle. The average value of six analyses was 0.023 per cent. This figure is very much higher than that obtained by Boyd (1921), who estimated that the muscle contained 0.0045 per cent magnesium, expressed as a percentage of the dry weight. Since the muscle contains about 80 per cent of water, this represents a concentration in the fresh muscle in the order of 0.0009 per cent. McCance and Widdowson (1940) reported that the flesh of boiled lobster, presumably the European lobster, *Homarus vulgaris*, contained 0.034 per cent magnesium. The flexor muscle of the American lobster, boiled for twenty minutes in three per cent sodium chloride solution, was estimated by the author to contain 0.022 per cent magnesium.

IN PICKLE

Estimations of magnesium in the pickle of commercially canned lobster are given in table I, in conjunction with the analyses of ammonia. The wide range in the magnesium content, from 0.010 to 0.028 per cent, is undoubtedly attributable to causes outlined below.

EXPERIMENTAL LOBSTER CANNING

There are three steps in the canning of lobster in which sea-water or fresh water may be used. The lobsters may be steamed, boiled in fresh water (with or without the addition of sodium chloride) or boiled in sea-water (with or without sodium chloride added). The meat may be washed in sea-water or in fresh water. Finally, in the preparation of the pickle a sodium chloride solution may be used, sea-water may be used alone, or sea-water to which sodium chloride has been added. Ordinarily, approximately one ounce (28 ml.) of pickle is

TABLE III. Effect of sea-water on magnesium concentration in canned lobster. Experimental pack, six months old. A plus sign indicates that sea-water was used.

Sea-water in			Grams per 100 ml. of pickle		
Cooking	Washing	Making pickle	Magnesium	Ammonia nitrogen	Crystals
−	−	−	0.013 0.015 0.0155	0.043 0.029	None Only one or two None
+	−	−	0.015 0.020	0.023 0.028	None None
−	+	−	0.0175 0.029 0.018	0.025 0.04 +	Very many Few on lining Many
−	−	+	0.018 0.013 0.022	0.033 0.04 + 0.028	Very many Many Few
+	+	−	0.031 0.022	0.04 + 0.039	Only one or two Several larger
+	−	+	0.020 0.019	0.027 0.032	Many on lining Few
−	+	+	0.033 0.029 0.028	0.021 0.032 0.025	Many large Few small Several large
+	+	+	0.028	0.041	A number

added to a "half-pound" can. Incidentally, many lobster canners prefer to use sea-water in one or more of the above steps, maintaining that the use of sea-water enhances the flavour and improves the colour. Controlled canning experiments carried out at this laboratory have failed to provide any confirmation for this theory.

In order to determine the effect of sea-water on the concentration of magnesium in the canned product, canning experiments were conducted in which sea-water and fresh water were alternatively and conjointly used in the canning procedure. The various combinations are outlined in table III, and the concentration of magnesium and ammonia in the pickle is also given. If we disregard, for the moment, the formation of magnesium-ammonium-phosphate crystals, the formation of which must have reduced the concentration of magnesium in the pickle and the meat, it will be seen from the table that the use of sea-water in any step of the canning procedure increased the concentration in the final product. This was most evident where sea-water was used for washing the meat and in the making of pickle. This was to be expected, since sea-water of 3.5 per cent salinity contains 0.132 per cent magnesium, in contrast to that of lobster meat with a magnesium content of 0.023 per cent. The minimum concentration of magnesium in the pickle of cans of known history, in which no sea-water was used in the canning process, was 0.013 per cent, the maximum was 0.017 per cent, and the average 0.015 per cent. It will be seen from the table that the concentration of magnesium in the pickle was often more than doubled by using sea-water in at least two steps of the canning procedure.

Influence of Sea-Water

The relation between "struvite" formation in the canned product and the use of sea-water in the canning process is shown in figure 1. The lobsters were canned under controlled experimental conditions at this laboratory, and the cans were examined at the ages indicated. The graph is based on the results of periodic examinations of some 240 cans of lobster, and for purposes of clarity has been divided into two sections. The amounts of magnesium-ammonium-phosphate crystals deposited on the lining and the meat were determined subjectively. A numerical value of 15 was given to those cans in which large amounts of crystals had formed, and other cans were rated between 0 and 15 depending on the extent of crystal formation. Considerable individual variation was found in the amount of crystals formed among various cans of the same group and of the same age. The points of most of the curves are far too scattered to permit accurate graphical construction. The curves, therefore, indicate trends rather than precise, mathematical rates of magnesium-ammonium-phosphate crystal formation in the various lots.

Fewest crystals were formed in those lots in which no sea-water was used at all, or in which sea-water was used only in the cooking of the lobsters. It is interesting to note that the incidence of crystal formation was slightly greater in the control A than in the lot in which the lobsters were boiled in sea-water. This suggests that the use of sea-water for boiling the lobsters has a slight inhibiting action on the formation of crystals within the can. The evidence, however, from the limited number of cans examined is far from conclusive, and in general the only difference between the two groups was the formation in group A of a few very small crystals while none had formed in lot B. These differences were less marked in the last examination after thirteen months' storage. Most significant is the fact that there was no sharp increase in crystal

formation during storage in both of these groups, and that after thirteen months' storage there were, on an average, only a few small crystals on the lining of the cans. Curves C and D indicate that the washing of meat in sea-water induces crystal formation in canned lobster to approximately the same degree as the use of sea-water in making the pickle. A considerable quantity of crystals had formed on the lining and the meat after a period of six months, but the increase after that time was very limited. The quantity of "struvite" deposited in the cans when sea-water was used in two steps of the canning process was

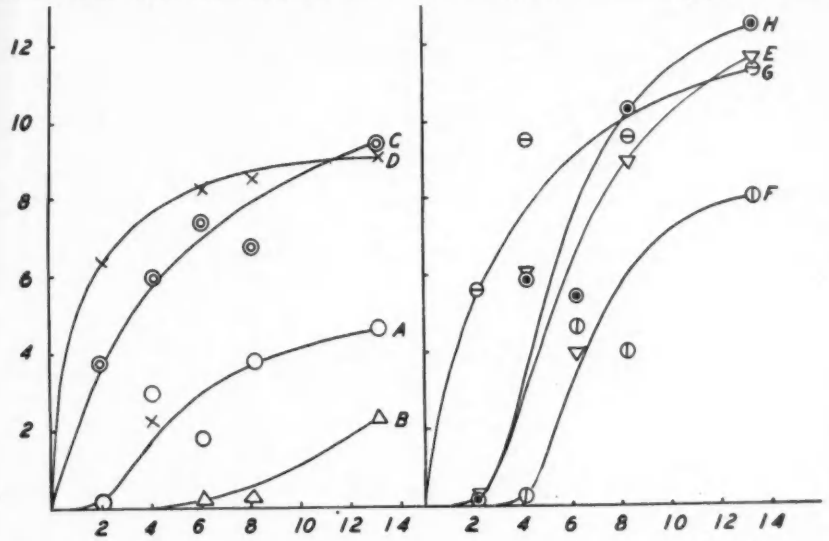

FIGURE 1. The effect of sea-water on the formation of struvite in canned lobster. Ordinates represent the relative amounts of struvite formed; abscissae represent the time of storage in months. A—No sea-water used. B—Sea-water used for cooking. C—Meat washed in sea-water. D—Pickle made with sea-water. E—Lobsters boiled in sea-water; meat washed in sea-water. F—Sea-water used for cooking, and for making pickle. G—Meat washed with sea-water; pickle made with sea-water. H—Sea-water used throughout.

somewhat greater than when sea-water was used in one step only. This is illustrated in curves E and G. However, the degree of crystal formation in canned lobster in which sea-water was used both in the boiling of the lobsters and in the making of the pickle, curve F, was appreciably less than when it was used only in one of these steps. Again in curve H, where sea-water was used throughout the canning procedure, the rate of crystal formation during the first four months after canning was definitely less than that indicated by curve G. This lends weight to the suggestion already made that boiling lobsters in sea-water inhibits crystal formation during the early months of storage.

Some 570 commercial cans of lobster of known history were also examined. It was noted that not more than traces of crystals had formed in those cans in which sea-water had not been used, but that many crystals were deposited in cans in which sea-water was used in two or more steps.

It is of interest to note that experimental canning of lobster conducted some years ago by Dr. E. Hess of this laboratory (unpub. data) entirely confirmed the finding outlined above.

It must be concluded, therefore, that the amount of magnesium-ammonium-phosphate crystals formed in canned lobster is influenced by the use of sea-water. In view of the relatively high magnesium content of sea-water as compared with that of lobster muscle, it would appear that the degree of crystal formation in canned lobster is limited by the concentration of magnesium.

TABLE IV. Formation of "struvite" crystals in canned lobster on addition of ammonia, magnesium, or magnesium plus ammonia to the pickle.

Treatment	Crystals after the following periods				
	2 weeks	4 weeks	7 weeks	10 weeks	32 weeks
Control					
Added one ounce (28 ml.) of pickle containing 7% NaCl.	None	—	None	—	A number
M					
Added one ounce of pickle containing 8% NaCl, plus 1% Mg as $MgSO_4 . 7H_2O$.	None	Several large clusters	*Very many*	*Very many*	*Very many*
N					
Added one ounce of pickle containing 8% NaCl, plus 1% ammonia N as $(NH_4)_2SO_4$	A number	Very few	Very few	Very few	Very few
P					
Added one ounce of pickle containing 8% NaCl, plus 1% Mg as $MgSO_4 . 7H_2O$, plus 1% ammonia N as $(NH_4)_2SO_4$.	*Very many*		*Very many*	*Very many*	*Very many*

In order to determine to what extent the formation of crystals is limited by the concentration of available ammonium and magnesium ions, a further canning experiment was conducted (table IV). Four groups of five cans each were put up. In one group magnesium sulphate was added to the pickle, in a second group ammonium sulphate was added, magnesium sulphate plus ammonium sulphate was added to a third group, and the fourth lot was used as a control. The lobsters were steamed for about 15 minutes, the meat was washed in fresh water, and 8 per cent sodium chloride was used for pickle. It will be seen that large quantities of crystals formed only in those cans to which magnesium, or magnesium plus ammonia, were added. It is significant that very few crystals, approximately comparable in amount to those found in the controls, were formed in the cans to which ammonia alone was added. Therefore, both inorganic phosphate and ammonia are present in canned lobster in sufficient

concentration for the formation of large quantities of magnesium ammonium phosphate, and the extent of crystal formation is limited by the concentration of magnesium in the canned product. Analytical data already presented point to the same conclusion.

DISCUSSION

The solubility of hydrated magnesium ammonium phosphate in distilled water is stated to be 0.0231 per cent at 0°, 0.052 at 20° and 0.0195 at 80° C. (Hodgman 1937). Its solubility in lobster pickle will be influenced by a number of factors, among them being the hydrogen-ion concentration, presence of ammonium salts, and very probably by the presence of protein breakdown products of which there are considerable amounts in the pickle. To obtain tentative values for the solubility of magnesium ammonium phosphate in lobster pickle, cans of lobster which had been stored from one to two years were alternately incubated at 25 to 30° C. and cooled at 15° C. over a period of several weeks. This should tend to hasten precipitation of the crystals. If the treatment resulted in the maximum precipitation of magnesium ammonium phosphate, it is a reasonable assumption that further precipitation was prevented by the lack of some component ion (assuming no change in pH or in the chemical constituent of the contents). In other words, in a solution containing magnesium, ammonium and phosphate ions, precipitation of $Mg=NH_4PO_4 . 6H_2O$ will continue until equilibrium is reached between the precipitate and the solution of the constituent ions. Further precipitation can take place only by an addition to the solution of that ion which limits precipitation (pre-supposing an excess of the two other ions). It was found by analysis that the minimum concentration of magnesium in the pickle of canned lobster, treated as outlined above, was 0.010 per cent, and that the range in concentration was from 0.010 to 0.012 per cent. The variation for ammonia nitrogen was from 0.017 to over 0.04 per cent, with a minimum concentration of 0.017 per cent in recently canned lobster. The concentration of inorganic phosphates ranged from 0.05 to over 0.1 g. phosphorus per 100 ml. of pickle. The concentration of magnesium in some of these cans to which ammonium sulphate had been added (table IV) was also estimated to be approximately 0.010 per cent. In terms of combining weights in the formula $MgNH_4PO_4 . 6H_2O$, the minimum ammonia nitrogen content of 0.017 per cent is equivalent to 0.03 per cent magnesium and the minimum concentration of 0.05 per cent of inorganic phosphates to 0.039 per cent magnesium. Thus the magnesium content of the pickle is one-third or less that of ammonia or phosphates, when expressed as combining weights. It is very apparent, therefore, that the extent of formation of magnesium-ammonium-phosphate crystals in canned lobster is limited by the concentration of magnesium in the pickle. On the basis of a magnesium content of 0.010 to 0.012 per cent, the solubility of magnesium ammonium phosphate in lobster pickle is about 0.10 to 0.12 per cent—approximately twice its solubility in distilled water at room temperature.

No attempt has been made to measure the effect of variation in pH, or of soluble protein and protein degradation products on the solubility of magnesium

ammonium phosphate. The pH of the pickle of some thirty-two commercial cans of lobster, as well as the pH of the pickle of most of the cans of the experimental pack, were determined by a Beckman pH meter. The pH was found to range between 6.75 and 7.25, with a mean value of 7.0. The pickle of about ninety per cent of the cans examined varied between pH 6.95 and pH 7.05. It is unlikely that such a limited variation in pH had an appreciable effect on the solubility of the crystals. The pickle is about equally buffered, though not strongly, on either side of neutrality.

It is apparent from the analyses that magnesium is a dominant factor in the formation of magnesium-ammonium-phosphate crystals in canned lobster, and that the extent of crystal formation is limited by the availability of magnesium. Any factor, or step in the canning procedure, which results in an increase in the concentration of magnesium in the canned product should cause an increase in the amount of crystals deposited. This has been entirely confirmed by the canning experiments.

CONTROL OF FORMATION

Prevention of crystal formation in canned lobsters can be accomplished by the lowering of the pH by the addition of acid, such as acetic or citric, to the point where crystals do not form. The addition of acid is regarded unfavourably and it should be resorted to only if all other methods are unsuccessful. A logical method of control is to ensure that one or more of the ions necessary for the formation of the crystals is present in minimal amounts.

It has been shown previously that the concentration of ammonia and inorganic phosphorus in canned lobster is sufficiently great for the formation in the canned product of large quantities of magnesium-ammonium-phosphate crystals. In contrast, there is normally present in lobster muscle sufficient magnesium for the formation of but small quantities of magnesium-ammonium-phosphate crystals, and in actual practice it has been found that, even after prolonged storage, the quantity is so small that it would not be observed by the average consumer. Effective control, though not the complete elimination, of magnesium-ammonium-phosphate crystal formation can be accomplished if the producer of canned lobster refrains from using sea-water both in the washing of the lobster meat and in the preparation of the pickle.

SUMMARY

Analyses of canned lobster pickle have indicated that the concentration of inorganic phosphates ranged from 0.05 to 0.012 per cent, that of ammonia nitrogen from 0.17 to over 0.4 per cent, and that of magnesium from 0.010 to 0.033 per cent. The variation in the magnesium concentration has been attributed to the use of sea-water in one or more steps of the canning procedure.

The magnesium content of pickle from canned lobster in which no sea-water had been used in the canning process ranged from 0.013 to 0.017 per cent, with an average value of 0.015 per cent.

The solubility of magnesium ammonium phosphate in canned lobster pickle was estimated to range from 0.10 to 0.12 per cent.

It was established that there are adequate amounts of inorganic phosphate and ammonia in canned lobster for the formation of large quantities of magnesium-ammonium-phosphate crystals, but that the amounts formed are limited by the concentration of magnesium.

Experimental canning has shown that the formation of crystals in canned lobster can be almost completely eliminated if sea-water is not used in any step of the canning procedure.

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Reduction of Trimethylamine Oxide by Bacteria

1. The Enterobacteriaceae

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ABSTRACT

The action of the bacteria included in the family Enterobacteriaceae on trimethylamine oxide has been studied. It has been found that trimethylamine production from trimethylamine oxide is characteristic of all the tribes of this family (650 cultures representing 74 species). The only exceptions have been within the genera *Shigella* and *Erwinia*. *S. dysenteriae* and *S. paradysenteriae* are unable to produce trimethylamine under the conditions used in the present work. Eight out of seventeen unidentified species of the genus *Erwinia* were likewise inactive.

INTRODUCTION

Advancements in the differentiation and classification of bacteria have been made, only as new and improved methods have been developed for their chacterization. The present study was initiated in the hope that it might provide a new method of value in the study of bacterial taxonomy.

The observation that bacteria possess the ability to reduce trimethylamine oxide to trimethylamine is a relatively recent one. (Ackermann et al. 1926; Claren 1938; Watson 1939; and Tarr 1939). Earlier, Ackermann et al. (1926) had shown that trimethylamine oxide could be considered as a hydrogen acceptor in the respiration of muscle.

The distribution of trimethylamine and its oxide in nature is of interest in that it may throw some light on its utilization by bacteria. The relatively high concentration of trimethylamine oxide in marine fish (Yoshimura and Fujise 1929; Hoppe-Seyler 1933; Beatty 1938) and the factors involved in its reduction to trimethylamine during fish spoilage have been thoroughly investigated (Beatty and Gibbons 1937; Watson 1939; Tarr 1939; Shewan 1942). Hattori and Hasebe (1937) have reported on the trimethylamine oxide content of a wide variety of meat products. Its presence in marine (Kapeller-Adler and Csato 1930; Kapeller-Adler and Vering 1931) and terrestrial plants (Steiner and Loffler 1929; Hanna, Vickery and Pucher 1932) has been noted. The fate of trimethylamine, trimethylamine oxide, choline and betaine in the animal body and in man has been the subject of a number of papers. (Langley 1929; Lintzell 1934; Fomin 1937; Davies 1938; Fuchs 1938; and Tarr 1939).

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The magnitude of the problem covered by the present title made difficult the choice of a starting point. Our selection of the family Enterobacteriaceae was an arbitrary one but in part was based on a short preliminary survey of several of the more common families.

EXPERIMENTAL

Selection of Cultures

Transfers of identified cultures were obtained from a number of laboratories. The generosity of the donors of the cultures is gratefully acknowledged. The cultures were checked for purity after the first transfer at this laboratory and at several times during the course of the investigation. They were maintained as stock cultures on nutrient agar slopes.

Trimethylamine Oxide

The trimethylamine oxide was synthesized following the procedure of Dunstan and Goulding (1899), and was recrystallized from alcohol twice before use. It has been noted that a very slight reduction to trimethylamine occurs when the oxide is autoclaved in the presence of reducing carbohydrates. Careful analysis has revealed that the amount formed in this way is insufficient to interfere with the test to be described below.

Medium

The medium used for the determination of ability to reduce trimethylamine oxide was constituted as follows: trimethylamine oxide, 0.1%; glucose, 0.25%; bacto-peptone "Difco", 0.5%; sodium chloride, 0.5%; magnesium sulphate, 0.1%; di-potassium phosphate, 0.1%; pH adjusted to 7.2 before autoclaving. Tubed in 5 ml. amounts.

This medium was found to be suitable for the growth of all cultures used in this study. There can be little doubt that a more complex medium would prove more satisfactory for certain of the species investigated, but such improved growth has been sacrificed in favour of a simplified medium in which there are fewer possibilities of undefinable factors influencing the results. The trimethylamine oxide level (0.1%) used in this medium was found to be high enough to give sufficient trimethylamine for the detection of reduction.

Determining Trimethylamine

The procedure outlined below is a qualitative modification of the method described by Beatty and Gibbons (1937). It depends upon the ready volatility of the trimethylamine (B.P. 3.5°C.) at 45°C. in alkaline medium.

To the culture tube at the end of incubation:

(1) Add 1.5 ml. 40% formaldehyde. Shake and let stand 3 minutes.
(2) Add 3 ml. of saturated potassium carbonate.

The tube is immediately closed with a one-hole rubber stopper which has been fitted with a short glass tube (5 mm. wide x 4 cm. long), slightly constricted at the lower end, and containing a small piece of absorbent cotton which has

been impregnated with bromthymol blue (Clark 1928) adjusted to pH 4.0 with 0.1 N sulphuric acid. The tubes are then incubated in a water bath at 45°C. for 30 minutes. If trimethylamine is present in the culture, it distils from the broth and condenses in the cotton, causing a shift of the indicator colour to the alkaline side. The formalin serves to bind any ammonia that may be present. The adequacy of the formalin for this purpose has been demonstrated by the findings of Benoit and Norris (1942).

Experiments have shown that culture medium to which has been added trimethylamine to a concentration of 0.0005 molar shows a positive reaction in 30 minutes under the conditions described above. The addition of ammonia to the culture medium, or production of ammonia by the culture during growth does not interfere with or alter the sensitivity of the test if formalin is added prior to the addition of the potassium carbonate. Control tubes of sterile medium were incubated and tested for trimethylamine production with each fresh lot of medium prepared. A negative reaction was always obtained.

Incubation Time and Temperature

To ascertain the most suitable growth temperature and incubation period for the detection of trimethylamine production, representative cultures were selected, with each of which six tubes of the trimethylamine oxide broth were inoculated. Three tubes of each culture were incubated at 25°C. and three others at 37°C. One tube of each culture from each temperature was removed and tested for trimethylamine production at 4, 14, and 28 days. The results are recorded in table I.

Table I. The influence of incubation time and temperature on the reduction of trimethylamine oxide by certain bacteria.

Genus	Number of strains tested	Trimethylamine production					
		at 25°C.			at 37°C.		
		Incubation time (days)			Incubation time (days)		
		4	14	28	4	14	28
Proteus	8	* +	+	+	+	+	+
Escherichia	8	+	+	+	+	+	+ −
Aerobacter	3	+	+	+	+	+	+ −
Eberthella	4	+	+	+	+	+	+ −
Salmonella	4	+	+	+	+	+	+ −
Shigella	5	+	+	+	+	+	+ −
Shigella	2	−	−	−	−	−	−

* + Denotes trimethylamine production
− Denotes no trimethylamine production
+ − Denotes very slight production of trimethylamine

It is evident that an incubation period ranging from 4 to 14 days is adequate for the genera tested. In the experiments to be recorded an incubation period of 8 days has been used. In the case of strains which gave doubtful results longer periods of incubation have been used, but in general cultures giving a negative test at 8 days failed to become positive on prolonged incubation. The cultures were grown at the generally accepted optimum temperature for each genus.

It may be noted that in certain cases at 37°C. cultures which proved to be positive at 4 and 14 days showed a negative reaction at 28 days. This result can be attributed to a slow distillation of the amine from the culture during prolonged incubation at this temperature.

TRIMETHYLAMINE OXIDE REDUCTION

Some 650 cultures representing most of the genera and species of the family Enterobacteriaceae were tested for their ability to reduce trimethylamine oxide, following the methods outlined above. The organisms were transferred from 48-hour agar slopes into the trimethylamine oxide broth. The data which follow are the results from at least three separate experiments run at intervals over a period of three months. It has been impossible to check the constancy of trimethylamine producing powers with all cultures over a prolonged period. It has been found, however, that over an interval of two years there has been no loss of reducing properties in stock cultures carried on media devoid of trimethylamine oxide. It seems safe to assert that this property is a constant one. No results are available to show that an organism may acquire the ability to reduce trimethylamine oxide.

Tribe Eschericheae

The species of the tribe Eschericheae that have been studied and the total number of cultures of each that were tested are listed below:

Escherichia coli	102
" *coli* (intermediate)*	85
Aerobacter aerogenes	103
" *aerogenes* (intermediate)*	17
Klebsiella ozaenae	1

*Slow lactose fermenting types.

Hope for a division of the genera of this tribe on the basis of ability or inability to produce trimethylamine has not been realized, since all species proved capable of reducing trimethylamine oxide to trimethylamine. It is felt that the cultures used are all typical of the tribe. They range from completely typical cultures of *Escherichia coli* through the intermediate group to typical *Aerobacter aerogenes*. The data based on results obtained from a large number of identified cultures seem sufficiently adequate to justify the conclusion that reduction of trimethylamine oxide to trimethylamine is a characteristic of the tribe Eschericheae.

TRIBE PROTEAE

All of the species of the tribe Proteae that were tested were able to produce trimethylamine. They were as follows:

Proteus vulgaris	23	*Proteus ichthyosmius*	6
" *mirabilis*	12	" "A" strains*	5
" *morganii*	12	" "Cope"†	11
" *hydrophilus*	5	" unidentified	1

*Stuart et al. (1943). †Cope and Kilander (1942).

It seems reasonable to conclude that trimethylamine formation from trimethylamine oxide is a characteristic of this tribe. Five strains of *Proteus hydrophilus* have been tested and found to be positive for trimethylamine production. If this species is to be transferred to the genus *Pseudomonas* as proposed by Guthrie and Hitchner (1943), then trimethylamine production may have differential value for this organism. It may be of interest to add that under the conditions used in the present work, the tribe Proteae attacked trimethylamine oxide more actively than most of the other tribes of the family.

TRIBE SALMONELLEAE

Considering the tribe Salmonelleae as a whole it has been found that trimethylamine production may be assigned as a tribal property. As so frequently happens in the study of bacterial taxonomy the exceptions to this general conclusion provided the results of greatest interest. The findings for the different genera are best considered individually.

1. GENUS *Salmonella*

In the genus *Salmonella* some forty-eight cultures have been tested representing thirty-nine species or serological types. They are listed below:

Salmonella cholerae suis	2	*Salmonella* sp. (Hvittingfoss)	1
" *schottmuelleri*	4	" sp. (Onderstepoort)	1
" *typhi murium*	1	" sp. (Cerro)	1
" " " (Mutton)	1	" sp. (Rubislaw)	1
" *abortivoequina*	1	" sp. (Tel-avi)	1
" *abortus ovis*	1	" sp. (Kerkee)	1
" *enteriditis*	2	" sp. (Carrau)	1
" " (Dublin)	1	" sp. (Urbana)	1
" " (Newport)	1	" sp. (Worthington)	1
" " (Limerick)	1	" sp. (Senftenberg)	1
" *paratyphi*	2	" sp. (Derby)	1
" sp. (Kentucky)	1	" sp. (Reading)	1
" sp. (Newport)	1	" sp. (Thompson)	1
" sp. (London)	1	" *pullorum*	1
" sp. (Poona)	1	" *hirschfeldii*	1
" sp. (Minnesota)	1	" *paratyphi A*	6
" sp. (Bredeney)	1	" *anatis*	1
" sp. (Ballerup)	1	" *typhi suis* (Poppe)	1
" sp. (Vi typhosa)	1	Total	48

The wide range covered and the constancy of the positive results for trimethylamine production indicates that trimethylamine oxide reduction may be considered as a generic characteristic.

II. GENUS *Eberthella*

All of the sixteen cultures representing the type species of the genus *Eberthella* produce trimethylamine.

Eberthella typhosa	10
" *typhosa* "H 901"	1
" " "O 901"	1
" " phage type B1	1
" " " " E2	1
" " " " F2	1
" " " " D2	1

The other listed species (Bergey 1939) of this genus have not been tested. Stuart et al. (1943) have pointed out the weakness in the present classification (Bergey 1939) of this genus.

III. GENUS *Shigella*

The genus *Shigella* (table II) has provided an exception to the general findings of the production of trimethylamine from its oxide by the family Entero-

TABLE II. The action of the tribe *Salmonelleae* on trimethylamine oxide. Genus IX—*Shigella*

Species	Number of cultures tested	Trimethylamine oxide reduction
S. dysenteriae (shiga)	5	negative
S. paradysenteriae (flexner)	7	"
S. paradysenteriae (strong)	1	"
S. gallinarum	1	positive
S. alkalescens	96	"
S. sonnei	15	"
S. madampensis	17	"
Total	142	

bacteriaceae. *S. dysenteriae* and *S. paradysenteriae* fail to activate trimethylamine oxide while the other representatives of the paradysenteriae group *S. sonnei*, *S. alkalescens*, *S.madampensis*, and *S. gallinarum* are able to produce trimethylamine. There is a definite gradation in these latter species in the rate of trimethylamine production. They may be arranged in the order of diminishing activity as follows: *S. alkalescens*, *S. gallinarum*, *S. sonnei*, and *S. madampensis*. It has not been determined if this order is the result of lower activity or if it is

to be attributed to less growth in the case of the slower species. The amount of trimethylamine produced by *S. madampensis* is just sufficient to give a positive test.

This result is in agreement with the division noted by Stuart et al. (1942) based on the Eijkman test in which it was found that *S. alkalescens* and *S. sonnei* produce acid at 45.5°C. while *S. dysenteriae* and *S. paradysenteriae* do not. These authors have suggested that their results may find explanation on the grounds of lower degree of specialization in the case of *S. alkalescens* and *S. sonnei*. The present results lend support to such a suggestion since they indicate that trimethylamine oxide reduction is characteristic of the other less specialized genera of the family Enterobacteriaceae.

Tribe Erwineae

Eight out of a total of seventeen cultures from the genus *Erwinia* were able to produce trimethylamine. No attempt has been made to follow a large number of species in this genus. The cultures that were used were of known plant pathogenicity. Our inexperience with this genus and the absence from our stock collection of a large number of identified species has prevented a more detailed study. The results are recorded here to show the general relationship to the family Enterobacteriaceae, and in the hope that they may stimulate the interest of those with greater experience than ours in dealing with this group.

Tribe Serrateae

Twenty-three cultures representing three species have been studied and all found to be positive for trimethylamine oxide reduction. The species tested include:

Serratia marcescens	20
" *indica*	2
" *plymouthensis*	1

Further work on the remaining species is necessary. It is possible to conclude from the present data that trimethylamine oxide reduction is characteristic of *S. marcescens* and probably of *S. indica* and *S. plymouthensis*.

SUMMARY

The bacteria included in the family Enterobacteriaceae have been tested for their ability to reduce trimethylamine oxide to trimethylamine. It has been found that this property is possessed by all of the genera included in this family. The only exceptions have occurred within the genus *Shigella* and the genus *Erwinia*. *Shigella dysenteriae* and *S. paradysenteriae* are unable to reduce trimethylamine oxide under the conditions used in the present study. The genus *Erwinia* also contains species which are unable to bring about this reduction.

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